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HEMOCOMPATIBILITY AND ENDOTHELIALIZATION OF ELASTIN AND COLLAGEN SCAFFOLDS: THE ROLE OF PLASMA PROTEINS, SHEAR STRESS AND INTEGRINS

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HEMOCOMPATIBILITY AND ENDOTHELIALIZATION OF ELASTIN AND COLLAGEN SCAFFOLDS: THE ROLE OF PLASMA PROTEINS, SHEAR STRESS AND INTEGRINS

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ABSTRACT

Cardiovascular diseases are the leading cause of death worldwide as reported by the World Health Organization. Biomaterials have been extensively used in blood-contacting applications as heart valves, stents, grafts and catheters. However failure due to thrombosis hinders the long term patency and potential of these devices. Purified elastin scaffolds derived from porcine arteries as potential vascular graft materials are being investigated in our laboratory. In this study, we investigated the influence of factors such as shear stress and the presence of plasma proteins on the platelet response, and endothelial cell retention on purified elastin scaffolds to evaluate their hemocompatibility.

Elastin scaffolds showed minimal platelet attachment and activation as compared to collagen scaffolds under physiologically relevant conditions – in the presence of shear and plasma proteins. Also, the absence of plasma proteins significantly augmented the platelet response to the underlying substrate, especially under shear. Contribution of fibrinogen adsorption towards platelet attachment was studied by Circular Dichroism. Although a higher amount of fibrinogen was adsorbed on elastin surfaces than collagen, the conformation of adsorbed protein was similar to native fibrinogen on both collagen and elastin surfaces. Therefore, with our testing conditions, the role of fibrinogen in contributing to the difference in platelet activity on these two scaffolds was not apparent. Plasma recalcification time reasserted thromboresistance of elastin scaffolds showing more than a two fold increase in clotting time as compared to collagen.

The purified elastin scaffolds showed good retention of endothelial cells in static cultures but when cells were plated under shear significantly lower number of cells were able to attach to elastin surfaces. Role of integrins in endothelial cell adhesion was studied by adhesion inhibition assays. Both αv and β1 receptor subunits exhibited a significant contribution to the endothelial adhesion process to elastin scaffolds as blocking these receptors by their respective antibodies significantly lowered the number of cells attached to elastin under static conditions.

Further studies are needed to delineate the effects of the adsorption of other plasma proteins such as vWF on elastin hemocompatibility. Our current studies indicate that endothelialization of elastin scaffolds could be improved by activating specific integrin receptors on the cells thereby promoting their interaction with the substrate. Overall, purified elastin scaffolds displayed superior hemocompatibility than collagen scaffolds and with better endothelialization could become potential candidates for vascular replacement.

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CHAPTER 1 - INTRODUCTION

Cardiovascular disease is the leading cause of death worldwide as per the World Health Organization [1]. As of 2006, CVD accounted for 1 of every 2.9 deaths in the United States, amounting to approximately 34.3% of the total mortality rate [2]. Hence there is an overwhelming need for the development of new approaches to treat cardiovascular diseases and to enhance the current healthcare options.

Biomaterials have been long used in cardiovascular applications as catheters, pacemakers, heart valves, stents, hemodialysis units, vascular grafts, ventricular assist devices and extracorporeal support systems. However, the performance of these devices has been significantly affected by biomaterial associated thrombosis. The associated complications include embolism, infarction, intimal hyperplasia, and occlusion leading to limited success or often complete failure of the device [3].

The search for the ideal non-thrombogenic material for biomaterial applications has been on for over three decades now. The process of blood-biomaterial interactions is highly complex; various factors including – platelets, plasma proteins, leukocytes, complement system and other mechanical factors play a significant role as described in Figure 1.1 [4]. Thrombosis, especially with cardiovascular devices remains a serious concern for long-term patency. The lack of availability of a suitable graft for small diameter vascular applications is partly due to the complications associated with thrombosis.

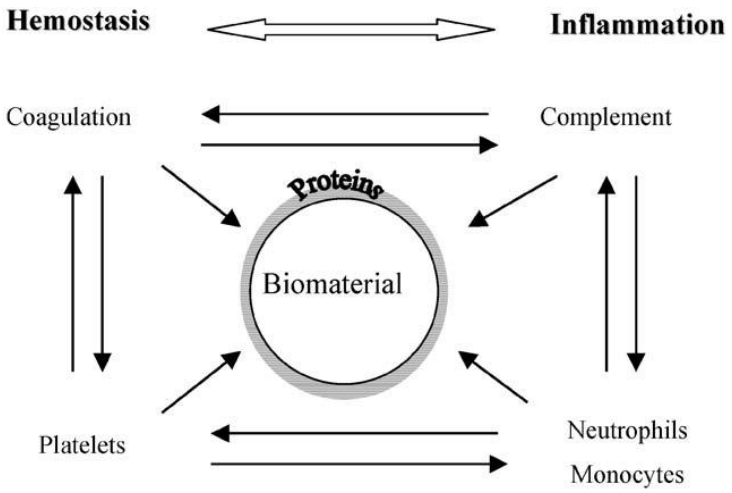

Fig 1.1: Schematic overview of the various cellular and plasma components involved in blood-biomaterial interactions [4].

The following chapter is an overview of the humoral and cellular components involved in the process of biomaterial-associated thrombosis and a summary of the various approaches currently adopted to improve the hemocompatibility of cardiovascular biomaterials.

Chapter 3 outlines the basis of the research project, specific aims, and the relevance to the broad goal of developing a hemocompatible biomaterial suitable for vascular graft applications. Chapter 4 describes the materials and methods employed and the results of the studies are presented in Chapter 5 along with relevant discussion.

The final chapter will summarize the observations and provide a comprehensive conclusion to the project with recommendations and scope for future research.

CHAPTER 2 – BACKGROUND

2.1 Biomaterial associated thrombosis – role of humoral and cellular components

2.1.1 Blood coagulation cascade

The coagulation cascade involves a series of proteolytic zymogen-enzyme conversions, which are self-amplifying and ultimately result in fibrin clot formation [4, 5]. The cascade comprises of two distinct pathways – intrinsic and extrinsic pathway.

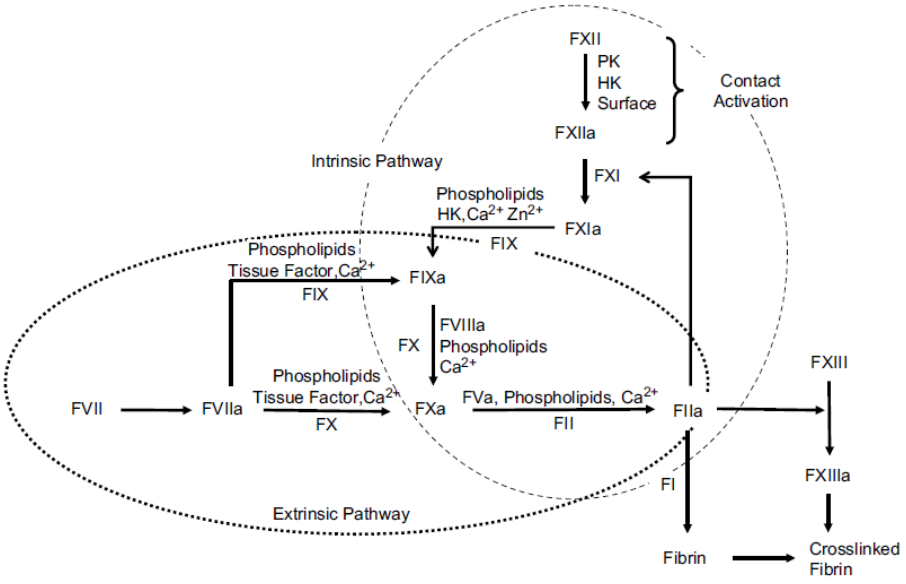

Fig 2.1: Simplistic schematic representation of the major pathways and enzymes involved in the blood coagulation cascade [5].

While the intrinsic pathway is triggered by the surface-mediated activation of Factor XII, the extrinsic pathway is initiated by the expression of Tissue factor (TF) by the damaged tissue [4]. The two pathways converge with the generation of thrombin,

which in turn mediates the conversion of the inactive fibrinogen to fibrin. The physiological relevance of the intrinsic pathway still remains under debate. However, there are indications of the role played by the intrinsic pathway in the propagation phase of coagulation, while the extrinsic pathway is responsible for its onset [4].

A schematic representation of the cascade is represented in Figure 2.1. In-depth review of the underlying mechanisms of the two pathways and the various mediators involved is beyond the scope of this report.

2.1.2 Plasma proteins and coagulation pathways

As soon as a foreign material is encountered by the body, the instantaneous response is protein adsorption [4, 6]. Ensuing reactions such as triggering of the coagulation cascade, platelet adhesion and activation, largely depend on the nature of the adsorbed proteins on the biomaterial surface.

The amounts of individual proteins and their conformational states are determined by the surface properties of the material, and these factors in turn influence the hemocompatibility of the material [3, 7]. Several factors including hydrophilicity or hydrophobicity, wettability, surface tension, charge density and smoothness have been implicated to play a role in modulating protein adsorption behavior [3, 7, 8]. In general, hydrophobic surfaces are shown to support strong interactions with adsorbed proteins. The heat generated during this process leads to the denaturation of these proteins ultimately causing conformational changes and subsequent accessibility of active sites for

the binding of corresponding receptors. Nevertheless, the procoagulant activity arising due to contact activation, is observed to be lower on hydrophobic surfaces [9]. Hence, more than the amount, the species and activity of specific proteins adsorbed at the interface, seem to modulate the biomaterial response.

During physiological wound healing processes, platelet attachment to exposed subendothelium is known to be mediated by plasma proteins – fibrinogen, Von Willebrand Factor (vWF) , fibronectin, thrombospondin and thrombomodulin [10]. Predictably, the proteins which are mainly involved in the interaction with biomaterials include fibrinogen, albumin, Hageman factor (Factor XII), high molecular weight kininogen (HMWK), collagen, fibronectin, von-Willebrand factor and these in turn are best studied in the context of material hemocompatibility [11-13].

Biomaterials are conventionally thought to set off the intrinsic pathway of the coagulation cascade, by causing auto-activation of Factor XII [1, 3, 5]. Negatively charged hydrophilic surfaces are said to increase the magnitude of this response, possibly by interacting with the positively charged amino acids of Hageman factor [3]. This activation also leads to the formation of an activation complex comprising of HMWK, prekallikrein and factor XI. During this process, the prekallikrein and factor XI come spatially closer, resulting in their reciprocal activation, further amplifying the coagulation response [1, 3]. HMWK actively participates in these reactions by complexation with prekallikrein and factor XI, thereby enabling enzymatic cleavage of these proteins by the activated factor XIIa. Substitution of adsorbed fibrinogen with factor XII and HMWK,

and subsequent activation have indeed been reported *in vitro*. Fibrin formation is directly correlated with factor XII activation. However, other evidence suggests that mere contact activation does not correlate with thrombin generation, indicating that contact activation is not the principal mechanism of biomaterial-stimulated plasma coagulation [14]. Also, recent studies have claimed that biomaterial-induced Factor XII activation is not surface-specific and does not play any role in furthering the coagulation response via the intrinsic pathway [9, 15].

Fibrinogen is an important player in the adsorbed protein layer on a biomaterial surface and is pivotal in initiating platelet response. Materials, whose adsorbed protein layer is dominated by fibrinogen, tend to exhibit a higher thrombogenic potential [7]. Typically, hydrophilic surfaces show a lesser amount of fibrinogen adsorption as compared to hydrophobic surfaces. Surface adsorbed fibrinogen is known to directly interact with platelets by means of the membrane glycoprotein receptor GPIIb/IIIa [11]. In their normal state, reversible interactions are shown to prevail between the platelet receptors and fibrinogen. However, it is important to note that plasma fibrinogen merely acts as a cofactor in this process, once the platelets are already activated [8]. The hypothesis proposed for this phenomenon is that adsorption unmasks regions that can form detectable complexes with platelet receptors [8]. Fibrinogen functions in bridging the gap between platelets and the biomaterial surface, and also between platelets during secondary irreversible aggregation [4, 8]. In some studies, adsorbed fibrinogen is also shown to be replaced with HMWK, thereby causing contact activation, over time [4].

Albumin is the most abundant protein found in plasma. It is one of the earliest proteins to adsorb onto a biomaterial surface, within milliseconds of exposure, owing to its high concentration and low molecular weight [16]. However, in most cases, albumin gets desorbed and substituted by other proteins that share high-affinity interactions with the surface, popularly known as the 'Vroman effect'[17]. Traditionally, albumin is considered to be non-adhesive to platelets due to the lack of established domains for platelet binding [18]. Many studies have proven that albumin surface-coatings confer superior hemocompatibility to biomaterials [19, 20]. Recent findings challenge this belief showing the existence of binding domains exposed due to material-mediated conformational changes of the protein [18].

2.1.3 Platelets

Platelets are anucleated cellular fragments, derived from the megakaryocytes of the hematopoietic stem cell lineage. They are generally discoid in shape with a smooth topography. When they get activated, they become spherical and extend pseudopodia to adhere to the agonist [12]. The glycocalyx present covering the plasma membrane of platelets houses a variety of glycoproteins that act as receptors for a number of adhesive molecules, aggregators and procoagulant factors [8, 12]. The membrane phospholipids are also known to promote coagulation by the conversion of Factor X to Xa and that of prothrombin to thrombin [12]. The cytoplasmic contents of platelets include mitochondria, lysosomes, peroxisomes, dense tubular system, α-granules and electron dense granules [12]. These intracellular organelles contain various hydrolytic enzymes

and potential mediators of platelet aggregation and leukocyte stimulation [21]. The primary function of platelets is to prevent blood loss during injury by hemostasis [4, 12]. They also participate actively in wound healing processes [8].

Platelets are known to get easily activated when they come in contact with any thrombogenic surface. Several factors can act as potential agonists of platelet activation like, plasma proteins- fibrinogen and thrombin, exposure of collagen following vascular injury, subendothelial matrix components, inflammatory cytokines derived from leukocytes, etc. Platelet responses can be classified into reversible and irreversible responses. The reversible responses include adhesion, change of shape and reversible aggregation , while, platelet release and secondary aggregation are irreversible [22].

Once platelets adhere to a biomaterial surface and get activated, a series of reactions follow. Bioactive compounds stored in the intracellular granules of platelets such as thrombospondin, β-thromboglobulin, ADP, calcium ions, serotonin, platelet factor 4 are released [21, 23]. Platelet factor 4 (PF4) is documented to exhibit antiheparin activity and thrombospondin mediates platelet-platelet recognition during secondary aggregation [24, 25]. P-selectin, a transmembrane glycoprotein that is present in the α-granules of platelets, fuses with the plasma membrane, thereby expressing it on the surface of activated platelets [26]. P-selectin aids in low-affinity interactions between platelets and various leukocytes subtypes. Some amount of soluble P-selectin is also released during this process [4, 26]. Platelet activation also liberates arachidonic acid, which further results in the production and release of thromboxane B2, endoperoxides

and prostaglandins [4] . These released endoperoxides act as a positive reinforcement causing increased expression of fibrinogen receptors, promoting platelet aggregation [27, 28].

Morphological changes are another hallmark response to platelet activation. Activated platelets show spread morphology, increasing their contact area with the surface, encouraging platelet-platelet interactions and aggregation. The associated cytoskeletal rearrangement enables centripetal distribution of fibrinogen receptors, and secretion of the contents of the intracellular granules [8, 12]. This rearrangement of the platelet plasma membrane also plays a role in providing a site for communication between its membrane phospholipids and complexes involved in the coagulation cascade. It is also believed to act as a physical barrier between the active coagulation factors and their native inhibitors [8].

Platelet microparticles (PMPs) are formed by exocytotic membrane budding of activated platelets [4]. PMPs are formed following material-contact of blood and are believed to be rich in certain procoagulant factors. They help in propagation of the thrombotic response by adhesion to fibrinogen, fibrin and aggregation with other platelets [29].

The initial interaction of platelets with biomaterial surfaces are regulated by its membrane receptors. Of the various receptors, GPIb and GPIIb/IIIa have been shown by several studies to have profound influence in platelet-biomaterial interactions [13, 30]. GPIb is a leucine-rich glycoprotein that mediates the von Willebrand factor (vWF) -

platelet interaction [13]. vWF is synthesized and secreted by vascular endothelial cells and also produced by platelets and stored in the cytoplasmic granules. Under exposure to shear stress due to blood flow, GPIb undergoes a conformational change, rendering it available for vWF binding [4]. The other receptor that is critical to biomaterial interactions of platelets is the constitutively expressed integrin receptor, GPIIb/IIIa. Apart from being expressed on the platelet membrane, these receptors are additionally stored in the α-granules and are translocated to the membrane during release phase of the intracellular substances. Normally when present, GPIIb/IIIa exists in its inactive form with a low-affinity binding capability for fibrinogen. The conformational shift post-activation makes this a strong binding, ultimately leading to platelet aggregation and leukocyte-platelet interactions [30]. Also, other extracellular matrix components possessing the RGD sequence also can bind to the activated receptor.

While it is widely believed that fibrinogen adsorption contributes majorly to platelet attachment, other possible *in vivo* mechanisms initiating this process remain poorly understood. It is often presumed that platelet activation with respect to biomaterials occurs due to thrombin generation as the culmination of the intrinsic coagulation pathway or by the release of ADP from stimulated blood cells [4, 6]. Contesting this notion, some researchers have reported failure of thrombin and kallikrein blockers in reducing platelet activation [31].

Though platelet adhesion and activation are considered to play a central role in the thrombotic response to biomaterials, other pathways involving complement-mediated

activation and platelet-leukocyte interactions have garnered interest in recent times. Studies have shown that inhibition of platelet adhesion or activation alone does not result in inhibition of the generation of PMPs or removal of platelets from circulation [4]. This further elucidates the need to understand that biomaterial associated thrombosis is a multi cell-regulated phenomenon.

2.1.4 Role of leukocytes and complement system

In the context of biomaterial contact, leukocytes could possibly influence a wide range of activities including platelet recruitment and activation, promoting further leukocyte adhesion and aggregation, fibrin formation and fibrinolysis [6]. Activation of leukocytes results in the upregulation of specific membrane receptors like CD11b and PSGL-1, which interact with GPIIb/IIIa and P-selectin respectively [4]. It is important to note that the latter two molecules are expressed in high levels on the surface of activated platelets. Fibrinogen is another common agonist for platelet and leukocyte adhesion to biomaterials. Activated leukocytes become sticky, strongly binding to damaged endothelial cells and material surfaces. It is also to be noted that granulocytes show preferential adsorption than lymphocytes, which could be explained by their endogenous procoagulatory effect and direct role in platelet aggregation [6].

The extrinsic pathway of blood coagulation initiated by tissue factor expression, has also been suggested to play a role following interaction with biomaterials. Biomaterial contact acts as a potential stimulus for TF expression by leukocytes [32]. This response could result in the binding of Factor X to CD11b resulting in thrombin

generation or assembly of the prothrombinase complex on the leukocyte cell membrane [4]. Another characteristic response following neutrophil and monocyte stimulation is the release of inflammatory and procoagulant cytokines such as – Interleukin 1 (IL-1), IL-6, Tumor Necrosis Factor alpha (TNF-α); arachidonic acid metabolites like leukotriene B_4, platelet activating factor (PAF) and reactive oxygen species [4, 33]. These mediators act as chemoattractants for leukocytes, cause activation of platelets and also help create a localized microenvironment which is protected from physiological inhibitor molecules [4]. Interestingly, platelet release molecules earlier mentioned, promote leukocyte activation. This forms a network of mediators which mutually enhance the activity of the other cellular components involved in response to a biomaterial.

Complement system is another important contributor in recognizing biomaterials as non-self and in the ensuing inflammatory response [4, 33]. It comprises of more than 20 plasma proteins and two pathways, classic and alternate which converge into the common terminal pathway. Both the pathways form distinct C3 convertases, resulting in the production of the C3a (anaphylotoxin) and C3b. The C3b generated is involved in the assembly of C5 convertase, which in turn cleaves C5 to produce C5a (anaphylotoxin) and C5b. C5b further takes part in the formation of the terminal complement complex (TCC) which causes cell damage and lysis [4].

With regard to biomaterial-associated response, surface-induced covalent binding of C3b by the alternate pathway is considered most relevant. Biomaterial surfaces are classified as activating or non-activating based on their affinity to bind Factor B over

Factor H. Those surfaces which support the interaction of Factor B with the surface-bound C3b are said to be activating, while those favoring Factor H interaction are non-activating [33]. Some studies also report the activation of classic pathway of the complement system in response to certain biomaterials [34-36].

The complement system provides another link between the platelet and leukocyte response to biomaterials. The derivates of the complement-derived C3a and C5a have chemotactic effects on circulating monocytes and neutrophils, promote the release of inflammatory cytokines and lysosomal enzymes and cause aggregation of the cells [6, 33]. These effects are attributed to the membrane expression of the alpha chain of the iC3b, inactive form of the C3b molecule of the complement system [33]. Inhibition of the complement activation *in vitro* has shown to significantly reduce leukocyte adhesion [37, 38].

Complement activation of platelets has been shown possible by several ways. Inhibitors of C1 complement protein which initiates the classic pathway have been successful in reducing platelet adhesion and microparticle release on polyethylene surfaces [39]. Platelets are shown to possess a receptor for C1q, which then induces P-selectin membrane expression and GPIIb/IIIa conformational change [4, 39]. Insertion of TCC into platelets has also been shown to increase its procoagulant activity [4].

2.1.5 Role of mechanical factors

Flow and its related parameters- wall shear stress and shear rate contribute to the mass transport of cells and proteins and their resultant interactions with the vessel wall or artificial surfaces [4, 40, 41]. Even in the absence of biochemical agonists, physical factors have been able to cause thrombosis. Wall shear rate can regulate reaction kinetics of several coagulation factors by governing the rate of removal or replenishment of the individual species [40]. Adhesion of platelets has shown positive correlation with shear rate. Similar response is also observed for Factor X activation by the TF-VIIa complex via the extrinsic pathway of blood coagulation [4, 40]. Conformational changes in the otherwise linear complex are possibly effected by flow [40] .

On the contrary, increasing rate of shear is shown to reduce fibrin deposition [4, 40]. In reference to thrombus formation, turbulence caused by the high shear regions is proposed to activate coagulation factors which deposit in the adjoining recirculation zones that experience low shear and promote coagulation [40]. Concentration of red blood cells is also believed to work in tandem with flow conditions, due to collision effect resulting in increased frequency of interaction with the material surface [40, 42]. Shear is also said to activate leukocytes, though highly dependent on testing conditions and materials tested [4].

Shear-induced activation of platelets has been reported by many groups [41, 43, 44]. Both magnitude and duration of shear seem to play a part in platelet activation [40]. Upon exposure to shear, vWF binds to the activated GPIb receptor on platelets,

subsequent expression of GPIIb/IIIa and liberation of platelet vWF [42]. The binding between these two molecules results in irreversible aggregation. Furthermore, flow also controls the distribution of platelet release molecules which are responsible for secondary aggregation and setting off the coagulation response [41]. Tissue damage due to shear in association with platelet agonists, synergistically act to accelerate thrombotic response, thus making it an important factor to account for, during hemocompatibility testing of biomaterials [42].

2.2 Strategies to improve hemocompatibility of cardiovascular biomaterials

Different approaches have been attempted to overcome the problems associated with thrombotic failure of cardiovascular devices. Since hemocompatibility is primarily modulated by the biomaterial surface rather than by the bulk properties of the material, introducing surface modifications to reduce thrombogenicity has been a popular option [16, 45]. These strategies can be broadly classified into - passivation of the artificial surface so as to obtain a non-reactive material that resists protein and platelet deposition; and biofunctionalization of the material using bioactive molecules that modify the host response to the material to minimize adverse effects and support functional regeneration [45, 46].

2.2.1 Surface passivation

Both organic and inorganic materials have been studied for surface modifications of vascular materials. Some of the inorganic materials investigated for vascular coating

applications include carbon, titanium oxide and oxynitride, iridium oxide [16]. Pyrolytic and graphitic carbon coatings have been performed on stents and vascular prostheses, with improved patency initially, however with poor or insignificant reduction of thrombosis after long-term implantation [47]. Diamond-like carbon (DLC) coated on heart valves and stents, shows preferential albumin adsorption over fibrinogen but with an observed increase in platelet adhesion [16]. Overall, surface irregularities and tendency of carbide formation have been suggested for the shortcomings observed with carbon-based coatings [16, 47]. In comparison, hydrophilic titanium oxide coatings fare better with low protein adsorption, inhibition of fibrinogen activation and they are also reported to have anti-inflammatory properties [16].

As mentioned earlier, hydrophobic materials generally show a higher protein adsorption and resultant conformational changes, whereas hydrophilic surfaces show reduced interaction with proteins. However, it must be noted that highly hydrophobic materials like ePTFE have been successful as vascular grafts in large diameter applications. Based on this general idea, hydrophilic molecules like polyethylene oxide (PEO), polyethylene glycol (PEG) and polysulfones have been used to modify synthetic polymer surfaces [1, 16, 45, 47-52]. The reduced interfacial free energy, lack of surfaces charges and steric repulsion are believed to be the reasons for the reduced protein adsorption observed with PEO [45]. Though many *in vitro* studies have been encouraging with minimal protein adsorption and platelet adhesion, *in vivo* results have been inconsistent with reference to thrombogenicity [4, 47]. Apart from functioning as non-fouling surfaces, PEG modification has also been extended to immobilize bioactive

molecules as spacers, shielding the molecule from the substrate while facilitating interaction with the corresponding receptors [16, 49, 51, 52].

Albumin is another molecule which has been sought after for passivating biomaterial surfaces. Being the most abundant plasma protein combined with its low molecular weight, it is one of the first proteins to adsorb onto any artificial surface [16]. Early studies showed that platelet interactions were far less with albumin as compared to other plasma proteins like fibrinogen and immunoglobulins [47]. Ever since, attempts have been made to covalently link albumin directly or modify surfaces so as to support preferential albumin adsorption [16, 19, 47]. While *in vitro* studies showed significantly decreased platelet response, clinical studies have not been able to produce favorable results [20, 53]. Limitations related to direct linkage include immunological concerns, difficulties with sterilization and shelf life, poor control over conformational changes in the protein structure [16]. Additionally, some recent studies have challenged the very idea of albumin being non-reactive with platelets [18].

Yet another interesting approach is derived from the lipid bilayer structure of the plasma membranes of resting erythrocytes and platelets. The outer lipid layer of these cells have been shown to consist of the same polar head group, phosphorylcholine [54]. Several attempts have proven that phosphorylcholine when incorporated to biomaterials through *in situ* polymerization, ozone grafting, heat stabilization or grafting directly into polymer backbone, show significant reduction in protein adsorption and platelet attachment [47, 55]. Initial clinical studies for phosphorylcholine-coated stents have

reported reasonable success, however these results were not reproduced in randomized trials [47, 56]. Another derivative, 2-methacryloyloxyethyl phosphorylcholine (MPC) has been successfully used as coatings on Dacron grafts and as part of copolymers, with favorable results in animal studies [47, 57, 58].

Elastin, an important structural component of blood vessels has shown to elicit minimal platelet response and anti-proliferative properties for smooth muscle cells [59, 60]. These observations have inspired elastin-based surface modifications of synthetic materials and development of electrospun and tissue engineered scaffolds for vascular applications. The innate difficulty of purification, processing and insolubility of elastin had limited its usability [47]. However, several functional sequences have been identified which has led to the development of elastin-mimetic peptides, polymers, and recombinant elastin sequences, which are now widely being used to provide non-thrombogenic coatings for cardiovascular biomaterials [47, 61-63]. Elastin peptides coated using surface impregnation and/or passive physical adsorption has improved the performance of small diameter vascular grafts and catheters in both *in vivo* and *ex vivo* studies [61, 63]. Furthermore, these surface modifications have shown to reduce the release of proinflammatory cytokines and inhibit smooth muscle cell migration, thereby making it an attractive approach to simultaneously prevent another looming problem with vascular devices, intimal hyperplasia [47, 59]. In addition, recent studies have also reported the use of elastin-mimetic triblock copolymers as protective surface coatings for small diameter ePTFE vascular grafts [62].

2.2.2 Biofunctionalization

The second kind of approach- biofunctionalization has employed pharmacological methodologies like the use of several established anticoagulants, anti-platelet drugs, direct thrombin inhibitors and bioactive molecules such as thrombomodulin and nitric oxide [1, 16, 47].

Heparin was the first anticoagulant to be immobilized to improve hemocompatibility of a surface [16]. Heparin in association with antithrombin III (AT III) binds with high affinity to its target molecules Factor Xa and thrombin, inhibiting the coagulation cascade [16, 64]. It has also been found to possess anti-inflammatory properties [16]. The methods used for modification range from adsorption, covalent grafting, use of albumin-heparin conjugates to end-point immobilization, which is currently used clinically [16, 47, 65-67]. However, the outcomes of *in vivo* biocompatibility of heparin-coated surfaces have been mixed with some *in vivo* results showing significant improvement and others showing no difference from uncoated counterparts [4, 47]. One of the inherent problems associated with surface-bound heparin is its multi-fold reduced bioactivity in comparison with free heparin [16]. Also, the short half-life and inactivation by systemic administration of protamine sulfate post extracorporeal circulation as in the case of hemodialysis, have affected the bioactivity of heparin conjugated to material surfaces [16, 47].

Other direct thrombin inhibitors both pharmacological and physiological have also been examined to improve biomaterial hemocompatibility. Hirudin and its

recombinant analogs, argatroban and benzamidine are a few inhibitor molecules which have been tested as surface coatings [16, 47]. Conjugation of recombinant hirudin with Dacron has yielded favorable *in vitro* and short-term *in vivo* results [68-70]. One advantage of employing direct thrombin inhibitors is that, they do not require the presence of antithrombin III which might be deficient in septic tissues [16]. Thrombomodulin is a molecule naturally produced by endothelial cells that not only binds to existing thrombin with high-affinity but also limits further thrombin generation by activation of anticoagulant protein C [16, 47, 71]. The molecule has been grafted covalently to polyurethane and ePTFE, and has shown promising *in vitro* and short-term *in vivo* outcomes [47, 72]. Nevertheless, the reduction in activity due to immobilization, cost factors, and unpredictable stability of conjugated thrombomodulin *in vivo* have limited its clinical applications [16, 47]. Alternatives like genetic engineering to produce modifiable and more stable thrombomodulin molecules are currently being explored at *in vitro* levels [73, 74].

Another common approach to improving biomaterial performance is the use of anti-platelet drugs, aimed particularly at inhibiting platelet activation and aggregation [16, 47]. Aspirin, an inhibitor of the production of arachidonic acid pathway platelet agonists, is the most widely used anti-platelet drug [47]. Inhibitors of platelet membrane receptor GPIIb/IIIa (abciximab) and molecules which inhibit platelet activity by stimulating an increase in intracellular cAMP levels including prostaglandins, prostacyclins, dipyridamole have also been considered for immobilization to biomaterials like polyurethanes, stents and Teflon grafts [47, 68, 75-77]. Aspirin-eluting materials and

coatings; stents coated with abciximab or releasing iloprost (prostacyclin analog) have shown promise with initial *ex vivo* and *in vivo* studies [75, 78-80]. However, one common limitation of drug-eluting or controlled release strategies is the recurrence of stenosis and the quantity of drug that could be loaded in the reservoir which limits the duration of effect [46, 47]. Nitric oxide (NO), another molecule released by vascular endothelium is known to be responsible for maintaining the vascular tone and vasodilation [71]. It has also shown to limit platelet activation and aggregation and also possess a negative effect on smooth muscle cell proliferation and neointimal hyperplasia [46, 47]. NO releasing coatings on hydrogels and films have shown release up to 2 months *in vitro* [47]. But the time limitations and problems related to harmful products leaching out, have led to research focusing on techniques that can produce nitric oxide from endogenous nitrites and thiol groups [81, 82].

2.2.2.1 Endothelialization

The endothelial lining which forms the blood-contacting surface of native vessels is the ideal, most non-thrombogenic surface known till date [1, 16, 45]. Hence, several efforts have been directed towards combining endothelial cells (EC) with biomaterials- either by means of *in vitro* cell seeding or by employing strategies to induce self-endothelialization of biomaterials upon implantation [16, 46]. One significant advantage of this approach is the ability to create a permanently potent hemocompatible interface [16].

Endothelial cells are known play an active role in regulating coagulation and thrombosis. EC synthesize a number of molecules, the relative expression of which governs thrombosis and fibrinolysis [71, 83, 84]. Activated EC show an upregulated membrane expression of TF, initiating the coagulation cascade. Apart from TF, several other factors such as vWF, platelet activating factor (PAF), tissue plasminogen activator (tPA), cell adhesion receptors are upregulated and number of other molecules including NO, plasminogen activator inhibitor-1 (PAI-1), thrombomodulin, prostacyclin (PGI_2), tissue factor pathway inhibitor (TFPI) are downregulated [71, 84]. These reactions cumulatively, promote coagulation and activation of platelets, leukocytes and the complement system. However in their native state, EC possess anti-thrombotic and protective effects essentially by a reversal of the above mentioned trend.

Some of the major negative feedback mechanisms of EC that control thrombosis are – thrombomodulin (TM) which inhibits thrombin generation, heparan sulfate bound to the EC membrane that binds to AT III with high affinity thereby inactivating thrombin, TFPI that is expressed on the EC surface and secreted extracellularly, release of vasoactive substances like endothelin, prostacyclin and nitric oxide which prevent platelet aggregation and maintain vasodilation [71, 84, 85]. All these features combined make endothelialization of biomaterials, an attractive approach to overcome problems related to hemocompatibility. It should however be noted that the endothelial cells must be maintained in their non-activated basal state to confer non-thrombogenicity to the underlying biomaterial [71] .

2.2.2.1.1 In vitro endothelialization

In vitro endothelialization of biomaterials is generally a two-step procedure beginning with harvest of autologous cells, expansion by cell culture, and seeding followed by implantation [1, 16]. Adult EC, pluripotent stem cells, or endothelial progenitor cells (EPC) are the common cell sources for endothelialization. Since EC do not readily adhere to synthetic materials, they are often pre-coated with ECM molecules like fibronectin and collagen [16, 45, 46, 86]. Studies conducted with human umbilical vein endothelial cells (HUVEC) and umbilical cord-derived progenitor cells show successful retention of seeded ECs even after administration of shear stress [87, 88]. *In vitro* seeding of EPC also proved to increase patency of vascular grafts with enhanced formation of endothelial lining *in vivo* [89]. Other techniques like coating polymer surfaces with titanium carboxonitride have also yielded better EC retention [90]. Such endothelial cell seeded vascular materials showed reduced platelet aggregation and better long-term patency in animal models [83, 91, 92]. Coculture with other vascular cell types like fibroblasts and smooth muscle cells also improve EC attachment to artificial surfaces [1]. However the differences in the substrate heavily influence EC behavior in terms of adhesion, proliferation, differentiation and migration [71]. Neither the one or two-stage procedures have been successful in clinical trials with the seeded cells getting washed off [1]. Additionally, the cost, labor, expertise and specialized facilities required for the time-consuming preconditioning of cells in bioreactors, potential contamination, uncontrollable changes in phenotype, and difficulties of obtaining enough quantities of autologous cells makes the *in vitro* approach less practical [1, 46, 85].

2.2.2.1.2 In situ endothelialization

In situ endothelialization of blood-contacting materials is an emerging strategy that precludes long culture times associated with *in vitro* cell expansion. They are also applicable for emergency requirements due to their immediate availability. EPC form the primary cell source for this approach. EPC are a small pool of cells derived from the bone marrow that are positive for CD133, CD34 and VEGFR-2 [85, 93]. While the latter two markers are retained, circulating EPC lose CD133 [93]. Therefore antibodies targeting CD34 and VEGFR-2 have been immobilized on stents and grafts in an attempt to home these progenitor cells [85, 94, 95]. Though endothelialization has been superior, adverse effects like intimal hyperplasia (IH) have been observed [46, 95]. This is assumed to be due to potential differentiation of EPC into vascular smooth muscle cell (VSMC) phenotype or due to the secretion of proangiogenic factors by EPC, that could support VSMC proliferation and migration [85, 96, 97]. Above all, the major limitation is the sparse number of CD34 positive cells that are EPC, and a lesser proportion that can differentiate into endothelial cells [98]. On the other hand, antibodies directed against VEGFR-2 possess good binding efficiency only at sub-arterial shear rates [85]. Current research is focused on conjugating VEGF to implant surfaces or use gene transfer to locally express VEGF (to avoid associated IH) [16, 85, 99]. One common difficulty with using EPC is their extremely low number in circulation. Several factors have been considered to improve mobilization and homing of EPC for this purpose. Pre-stimulation of the progenitor cells with high-mobility group box-1 (HMGB-1), leptin, stromal cell

derived factor -1 (SDF-1), activating integrin β_2 antibody before *in vivo* application has proven successful in ensuring EPC homing to sites of injury and ischemia [85].

ECM molecules, ligands and peptide sequences are often used to attract EPC to a biomaterial. Although whole ECM molecules like fibronectin, collagen and vitronectin promote EC attachment; they also possess domains for interaction with platelets and other inflammatory cells [16, 46]. Hence, presenting specific well defined domains of ECM molecules to promote endothelial adhesion is a common approach for endothelialization. Of the synthetic peptides, the RGD sequence first identified with fibronectin is the most widely used. Cyclic RGD (cRGD) shows better affinity to EC and EPC than linear RGD, as it more closely resembles its native conformation [1, 46, 85]. This interaction is mediated by integrin receptors. Several integrin receptors have been identified on EPC as being responsible for their adhesion and migration [46]. Incorporation of cRGD as a stent coating has shown improvement in endothelial cell recruitment both *in vitro* and after 12 weeks of *in vivo* implantation [100]. Interestingly, cRGD also acts as an antagonist for platelet membrane receptor GPIIb/IIIa [46, 101]. One potential limitation of this approach could be the presence of RGD-binding domains on other plasma proteins and cells, which can competitively bind to the peptide interfering with EPC capture [85]. Laminin-derived YIGSR peptide has been combined with PEG and NO to improve long-term patency of polyurethane grafts [102, 103].

Using magnetic labeling to home EPC has been tried with metallic stents and magnetized grafts [104, 105]. This process involves the *in vitro* culture of isolated EPC,

labeling with superparamagnetic microspheres followed by *in vivo* application [85, 105]. Though this method will offer well characterized EPC, it is a time and labor-intensive approach, whose potential long-terms effects are largely unknown [85]. Another novel approach proposes the use of aptamers, single-stranded nucleic acid sequences [1, 85]. These molecules can be selected with high specificity *in vitro*, can bind to cell-specific target sequences and also resist non-specific protein adsorption [85, 106]. Their efficacy as capture molecules has been demonstrated *in vitro* under shear [107]. *In vivo* performance of aptamers in homing EPC remains to be evaluated.

CHAPTER 3 – PROJECT RATIONALE

Elastin is the principal ECM component of the muscular medial layer of the vessel wall. It has been shown to be thromboresistant and has anti-proliferative properties for smooth muscle cells [59, 60]. Hence, it could potentially be a valuable material for substituting currently used synthetic grafts, especially in small diameter vascular applications, where synthetic grafts have shown limited success due to complications associated with thrombotic failure and intimal hyperplasia. Elastin-based and elastin-mimetic coatings have recently been investigated for their potential to improve biomaterial hemocompatibility, although clinical studies are yet to be conducted [47, 61, 62].

In our laboratory, we have earlier developed a purified elastin scaffold for vascular tissue engineering, derived from porcine tissue [108]. The scaffold offers the advantages of maintaining the native structural architecture of the vessel wall, provides good mechanical properties like compliance and porosity required for cellular infiltration and subsequent remodeling [109-111].

The broad objective of the present study is to study the platelet response and integrin-mediated early endothelial adhesion to purified elastin scaffolds. Any material used for blood-contacting applications should be tested for its hemocompatibility. Platelets play a central role in hemostasis and resulting thrombus formation; therefore platelet response to biomaterials needs to be studied for cardiovascular applications. Several groups have used platelet adhesion and activation profiles to assess the

hemocompatibility of biomaterials [12, 41, 65-67, 81, 112-115]. Other significant factors such as presence of plasma proteins and shear have also been incorporated into *in vitro* hemocompatibility testing systems [41, 64, 65, 116, 117].

Various methods have been investigated to improve the hemocompatibility of existing materials. Among them, promoting endothelialization is widely researched [46, 85, 89, 93]. Different ways to enhance adhesion and retention of endothelial cells using ECM ligands and peptides for EC membrane receptors are being studied in this context [100, 102, 103, 118]. Hence, minimizing platelet response and encouraging endothelial attachment could lead to improved biomaterial hemocompatibility.

Specific aims

Aim 1: To study platelet behavior on purified elastin scaffolds.

We would characterize platelet attachment and activation to tissue engineered elastin scaffolds using lactate dehydrogenase (LDH) assay and Scanning Electron Microscopy (SEM). Collagen scaffolds, also purified from porcine carotid arteries would be used as the positive reference material, since both are porous, protein-based, tissue engineered matrices thereby being highly relevant to study their comparative response. The significance of factors such as scaffold dimensions and porosity, plasma proteins and shear would be analyzed with respect to platelet behavior to our scaffolds. We also intend to understand how plasma proteins respond to the two different scaffolds by evaluating

plasma recalcification and also by studying surface-specific adsorption using Circular Dichroism.

Aim 2: To investigate endothelial cell retention and attachment to tissue engineered elastin scaffolds.

We would study the retention of endothelial cells seeded on elastin scaffolds under static and shear conditions at early time points. Also, since integrins are the membrane receptors primarily involved in EC adhesion to ECM molecules, we intend to study the role of specific integrin receptors in mediating adhesion to tissue engineered elastin scaffolds. We would evaluate the role of individual receptors in contributing to early-adhesion of endothelial cells to the substrates, as this is considered critical for maintaining non-thrombogenicity of the underlying material. The pattern of the relative importance of the different integrin receptors would be compared to collagen and plain tissue culture polystyrene. The knowledge obtained from this study could then be used to activate specific receptors on the endothelial cells to improve adhesion onto elastin scaffolds or even incorporate, increase the spatial distribution of well-defined ligands for specific integrin receptors for better attachment and retention of seeded endothelial cells on biomaterial surfaces.

CHAPTER 4 – MATERIALS & METHODS

4.1 Preparation of 3D elastin scaffolds

Porcine carotid arteries were obtained from Animal Technologies Inc., Tyler, TX, washed first with saline and rinsed thoroughly in PBS. The arteries were cleaned off the excess fat, surrounding fibrous tissue and then rinsed with PBS. Purified elastin scaffolds were obtained by cyanogen bromide (CNBr) treatment [119]. Briefly, arteries were allowed to stir in a solution of 70% formic acid (v/v) containing 50 mg/mL of cyanogen bromide for 24 hours (both chemicals were obtained from Acros Organics, Morris Plains, NJ). The treated arteries were heated at 60^{o}C for 1 hour followed by boiling for 5 minutes to inactivate CNBr. The purified elastin scaffolds were washed extensively in 70% ethanol (v/v) to remove the residual acid and stored in 70% ethanol.

4.2 Preparation of 3D collagen scaffolds

Carotid arteries obtained from the same source, were first decellularized, crosslinked and then elastin was removed to give purified collagen scaffolds, as described by Lu et al [108]. Decellularization was performed using sodium dodecyl sulfate (SDS) followed by trypsin digestion. Arteries were incubated in 1% SDS (w/v) solution in 10 mM Tris buffer (pH 8) for 4 days at room temperature. Samples were rinsed twice with Tris buffer, and digested with 0.05% trypsin (w/v) (Sigma-Aldrich Corp., St. Louis, MO), 0.1% ethylenediaminetetraacetic acid, EDTA (w/v) in 10 mM Tris buffer, pH 8, for 18 hours at 37^{o}C. The second step of crosslinking was done using 20 mM 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide (EDC) and 10 mM N-hydroxy succinimide (NHS) (Thermo Fisher Scientific, Rockford, IL) in 50 mM Hepes buffer at pH 6.5 at room temperature for 72 hours. Samples were later rinsed with 100 mM NaH_2PO_4 to quench residual EDC. Finally, elastin was removed by elastase digestion. 20 U/mL elastase (Elastin Products Company Inc., Owensville, MO) solution was prepared in 100 mM Tris buffer (pH 7.8) containing 1 mM calcium chloride and 0.02% sodium azide (w/v). Decellularized and crosslinked artery samples were incubated at 37^{o}C for 4 days. All steps were done with gentle shaking and using 4 mL solution per cm^2 of artery, but for the elastase digestion for which 2 mL solution was used per unit area. Purified collagen scaffolds thus obtained were rinsed well and stored in 70% ethanol (v/v). Also, the scaffolds were punched into desired sizes (to fit into 96-well microplate) before digestion with elastase.

4.3 Characterization of 3D scaffolds

4.3.1 Histology

Fresh carotids, purified collagen and elastin scaffolds were embedded in OCT (Tissue-Tek - Electron Microscopy Sciences, Hatfield, PA) and cryosections of 6 µm thickness were obtained. Masson's trichrome and Verhoff's Van Giesen (VVG) staining was performed to confirm decellularization and the presence of purified collagen and elastin respectively. Additionally, removal of glycosaminoglycans (GAG) and decellularization was checked by alcian blue staining on elastin scaffolds. All sections

were fixed in 95% ethanol (v/v) for a minute and washed in distilled water before staining.

For Masson's trichrome, samples were first fixed in Bouin's fixative and then successively stained in Weigert's Iron hematoxylin, Biebrich scarlet acid fuchsin and counterstained with light green S.F yellowish solution. For VVG staining, sections were placed in Verhoff's working solution, followed by ferric chloride treatment and counterstained with Van Giesen's solution. All staining solutions were obtained from Poly scientific, Bay Shore, NY.

In the alcian blue method, fixed samples were stained with 1% alcian blue (w/v) solution in 3% acetic acid (v/v) (pH 2.5) and counterstained with nuclear fast red. All slides were coverslipped with Permount (Fisher Scientific Inc., Pittsburgh, PA) and observed under the microscope (Zeiss Inc.).

4.3.2 DNA quantification assay

The removal of DNA from the purified elastin and collagen scaffolds was confirmed quantitatively by the DNeasy blood & tissue kit (Qiagen Inc., Valencia, CA) as per the manufacturer's protocol. Briefly, samples of fresh carotids and purified protein scaffolds were cut into small pieces and incubated in buffer containing proteinase K overnight at 56^{o}C with gentle shaking (n = 4 per group). DNA was purified using a column separation method and the absorbance measured at 260 nm in a UV/Vis

spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). DNA quantity was calculated as per the given instructions.

4.4 Preparation of collagen and elastin 2D scaffolds

Collagen and elastin two-dimensional scaffolds were prepared on glass coverslips (Electron Microscopy Sciences Inc., Hatfield, PA) 5 mm in diameter that fit into 96-well microplates. The glass coverslips were thoroughly washed with piranha solution (70% sulfuric acid and 30% hydrogen peroxide, both (v/v)) followed by a modified RCA (Radio Corporation of America) basic wash (1:1:3 v/v NH_4OH:H_2O_2:H_2O) to remove both organic and inorganic impurities, sonicated in ethanol and blow-dried with N_2 before coating with collagen or elastin peptides.

Rat tail tendon type I collagen (BD Biosciences, San Jose, CA) diluted in 0.02 N acetic acid was coated at a concentration of 1 mg/mL for 1 hour at room temperature. The coverslips were then washed thrice with PBS to remove excess acid. Elastin peptides (Elastin Products Company Inc., Owensville, MO) which contains a random mixture of peptides with the functional VGVAPG sequence, was dissolved in 0.01 N sodium acetate buffer (pH 5) containing 0.05% sodium azide (w/v), at a concentration of 10 mg/mL. The coverslips were incubated in this solution for 1 hour at 37^{o}C and allowed to dry overnight in a sterile hood. It is important to mention that for the platelet attachment studies, the 2D surface coatings were directly laid on the surface of the TCPS well plate.

4.5 Characterization of 2D scaffolds

4.5.1 Contact angle analysis

The air-water contact angle of the coated and the uncoated coverslips was measured with a CAM 200 optical contact angle/surface tension meter (KSV Instruments Ltd.). The plain, collagen and elastin peptide coated coverslips were mounted on the stage and contact angle obtained by dropping nanopure water on the surface. At least three measurements were taken per group. The protein-coated coverslips were washed twice with distilled water and blow-dried prior to taking readings, to get rid of non-specifically adsorbed surface impurities. Another set of measurements were also taken for the protein-coated samples, after hydration with nanopure water for an hour, to confirm if the adsorbed proteins were retained on the glass surface.

4.6 Preparation of platelet suspensions

Whole blood (30 mL) was collected from healthy volunteers by standard venipuncture procedure at the Redfern Health Center. It was made sure that all blood donors were not under any medication for at least a week prior to withdrawal. Blood was collected in BD Vacutainers containing acid citrated dextrose (ACD) (Becter-Dickinson, Franklin Lakes, NJ) and transported to the laboratory on ice. The first few mL of blood obtained was discarded as they tend to be high on clotting factors that may interfere with platelet studies. All protocols for participation of human subjects were approved by the Institutional Review Board at the University. Platelet suspension buffer (PSB) was

prepared with a composition of 137 mM NaCl, 2.7 mM KCl, 5.5 mM dextrose, 10 mM Hepes, 0.4 mM NaH_2PO_4 and 0.1 U/mL apyrase (Sigma-Aldrich Inc., St. Louis, MO) . 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$ was added to the PSB prior to platelet experiments to give appropriate metal ion concentrations.

Blood was centrifuged (Allegra 6R, Beckman Coulter, Fullerton, CA) at 1200 rpm for 15 minutes to separate platelet rich plasma (PRP) from the cellular components. PRP was carefully separated and the concentration of platelets present was measured using a particle count analyzer (Beckman Coulter, Fullerton, CA). The PRP obtained was used for platelet studies at a concentration of $1x10^8$ cells/mL. Platelet suspension buffer (PSB) with the metal ions, at pH 7.4 was used to dilute the PRP to the desired final concentration.

Washed platelets (WP) suspension was prepared using a gel separation technique. A liquid chromatography column made up of Sepharose 2B (Sigma-Aldrich Inc., St. Louis, MO) was allowed to equilibrate with PSB. PRP was pipetted into the column and buffer was allowed to flow through from a reservoir. Platelets being the high molecular weight components separated out first and were collected in microcentrifuge tubes. The turbid solution was collected until proteins separated out, indicated by a change in color of the solution. The platelet count in the pooled solution was measured and made up to a working concentration of $1x10^8$ cells/mL.

4.7 Platelet studies

Prior to platelet studies, the purified 3D elastin and collagen scaffolds were punched into circular discs that fit into 96-well microplates. The scaffolds were washed thrice with PBS and stored overnight in PBS before the experiments. Glass coverslips treated with Sigmacote (Sigma-Aldrich Inc., St. Louis, MO) was used as the negative control while collagen acted as the positive control. In case of 2D scaffolds, collagen and elastin peptides were directly coated on TCPS and glass-coated with sigmacote served as the negative control.

Orbital shear was provided using an orbital plate shaker (VWR, S-500) calculated using the equation,

$$T_{max} = a \sqrt{\eta\rho} (2\pi f)^3$$

(*a*- radius of gyration of the shaker (cm), *ρ*- density of solution (g/mL), *η*- viscosity at 37°C (poise) and *f*- frequency of rotation (rps)). Frequency of 250 rpm, as per the above equation corresponded to a maximal shear stress of 12.7 dynes/cm^2, comparable to arterial shear levels.

4.7.1 Lactate dehydrogenase assay

Platelet attachment was studied using lactate dehydrogenase (LDH) quantitative assay (CytoTox® Non-radioactive Cytotoxicity assay, Promega Corp., Madison, WI). 100 µL PRP or WP was added to each sample (n= 4-6 per group) and allowed to interact with the sample under static or shear conditions for 15, 60 minutes at 37^{o}C. After

incubation, the non-adherent platelets were removed by at least three washes with PBS. 100 µL PBS was added to each sample and adherent platelets were lysed with a lysis buffer. The amount of LDH present in each sample was measured following an enzyme-substrate specific colorimetric reaction. Absorbance was measured at 490 nm in a UV/Vis spectrophotometer (Bio-Tek Instruments Inc.) and the number of attached platelets was quantified using a standard curve constructed with samples of known platelet numbers.

4.7.2 Scanning Electron Microscopy

Platelet activation was studied by Scanning Electron Microscopy (SEM) with n=3 samples per group. Two and three dimensional scaffolds of elastin and collagen were treated with 100 µL of either of the platelet suspensions (PRP or WP) under static or dynamic conditions for one hour at 37^{o}C. Non-adherent cells were washed off with PBS thoroughly and the samples were fixed in Karnovsky's fixative containing 2.5% glutaraldehyde (v/v) and 2% formaldehyde (v/v) in 100 mM sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA) at pH 7.4 for 30 minutes at room temperature and rinsed again in PBS. Following this step, dehydration of the samples was performed using an ascending percentage-graded series of ethanol-water mixtures (50%, 60%, 70%, 80%, 90% and 100% (v/v)), for 10 minutes each. 20 µL of hexamethyldisilazane (HMDS) (Sigma- Aldrich, St. Louis, MO) was added to each sample and allowed to dry overnight in a chemical fume hood. Samples were observed using FESEM-Hitachi S4800 scanning electron microscope and images were taken. The morphology of the platelets

adhered was graded as described by Goodman et al on a five-point scale of increasing activation namely – round, dendritic (with early pseudopods), spread dendritic, spread and fully spread [120].

4.8 Measurement of plasma recalcification time

For measuring the clotting time of recalcified plasma, platelet poor plasma (PPP) was prepared by centrifuging PRP at 700g for 15 minutes and collecting the resulting supernatant. 100 µL of PPP was added to elastin and collagen 3D scaffolds placed in 2 mL microcentrifuge tubes (n=5 per group) and incubated at 37^{o}C for 1 minute. 100 µL of 25 mM $CaCl_2$ was then added and the solution was constantly monitored for the formation of fibrin threads by dipping a stainless-steel loop coated with silicone [121]. The first instance of fibrin formation was recorded as the clotting time. Standard glass coverslips were used as the baseline control.

4.9 Protein adsorption on elastin- and collagen-coated substrates

Quartz slides (0.375" x 1.625" x 0.0625", Chemglass) were cleaned with piranha solution, followed by a modified RCA acid wash (1:1:3 v/v HCl: H_2O_2: H_2O), sonicated in ethanol and dried using N_2 gas. The cleaned substrates were then coated with elastin peptides or with rat tail tendon type I collagen, as described previously.

The protein-coated surfaces were washed with distilled water to remove loosely bound protein and other impurities. Background Circular Dichroism (CD) and absorbance spectra for the collagen- and elastin-coated substrates were first obtained. The

slides were fully immersed in 25 mM potassium phosphate buffer (pH 7.4) in a four-well plate and a suitable volume of fibrinogen (FIB3, Enzyme Research Laboratories, South Bend, IN) depleted in Von Willebrand factor and fibronectin was added from the stock solution to give a physiologically relevant bulk concentration of 3 mg/ml. Care was taken to ensure that the protein was added below the air-water interface, to avoid denaturation of the protein. The slides were incubated in fibrinogen (Fg) solution for 1 hour at 37°C. After incubation, an infinite dilution step was performed to remove all the loosely bound and unadsorbed fibrinogen from the solution and also to avoid contact of the substrate with the denatured protein which may be present at the interface, had this dilution step not been performed. Finally, the slides were washed in nanopure water and taken for CD measurements to analyze the secondary structure of the adsorbed Fg.

4.10 Circular dichroism

The conformation of native and adsorbed fibrinogen (Fg) was studied by circular dichroism spectropolarimetry using a Jasco J-810 spectropolarimeter (Jasco Inc, Easton, MD) at room temperature. The native structure of Fg in solution was determined first, using a special high-transparency quartz cuvette (Starna Cells, Inc., Atascadero, CA) with a path-length of 0.1 mm. Fibrinogen was diluted from the stock to a known concentration using a 25 mM potassium phosphate buffer, pH 7.4. The background spectra consisting of buffer alone in the cuvette was measured, followed by the Fg solution. This background spectrum was later subtracted from the protein spectrum to account for the buffer effects.

The CD and absorbance spectra thus obtained were interpreted as described by Sivaraman et al [122, 123]. The ellipticity (in mdeg) was converted to molar ellipticity (deg•cm^2/dmol) by the following equation,

$$[\theta] = (\theta \bullet M_o)/(10{,}000 \bullet C_{soln} \bullet L) \qquad (1)$$

where [θ] is molar ellipticity, θ is ellipticity in mdeg, M_o is the average residue molecular weight (118 g/mol), C_{soln} is the Fg solution concentration (g/mL) and L is the path length of the cuvette (cm).

Since proteins have been shown to exhibit a peptide absorbance peak at 195 nm, the height of this peak (A_{195}) was used to construct a calibration curve of A_{195} vs. C_{soln} for different serial dilutions of Fg of known concentration [122, 124]. From Beer- Lambert's law we have,

$$A_{195} = \varepsilon_{protein} \bullet C_{soln} \bullet L \qquad (2)$$

where $\varepsilon_{protein}$ is the extinction coefficient of the protein in mLg^{-1}cm^{-1} (or cm^2/g), and L is the path-length of the cuvette. The slope of the above mentioned calibration curve is represented by the "$\varepsilon_{protein} \bullet L$" term from eq. 2.

It is important to note that the term "$C_{soln} \bullet L$" term in eq. 1 has units of g/cm^2, which is equivalent to the units for the surface concentration of the adsorbed protein. Based on the assumption that the absorbance of a protein is dependent on its mass per unit area in the path of the of the light beam, irrespective of whether the protein is in its

solution or adsorbed state, the calibration curve of A_{195} vs. C_{soln} may also be utilized for calculating the surface coverage of adsorbed Fg (Q_{ads}) [123].

Hence, the "$C_{soln} \bullet L$" term in eq. 1 can be replaced by Q_{ads}, to yield the following equation which was used for calculating the molar ellipticity of the adsorbed Fg,

$$[\theta] = (\theta \bullet M_o)/(10{,}000 \bullet Q_{ads}) \qquad (3)$$

At least three different measurements were taken for each substrate and the molar ellipticity vs. wavelength data obtained from the CD measurements were deconvoluted using the SP-22X algorithm and analyzed using SELCON software package to give percentage α-helix and β-sheet content of native and adsorbed Fg.

4.11 Cell culture

Human umbilical vein endothelial cells (HUVEC) were procured from Life Technologies Corp. Carlsbad, CA and cultured in Medium 200 supplemented with LSGS- low serum growth supplement. Cells between passage numbers of 3 and 7 were used in all the studies. Scaffolds used for endothelial cell studies were washed three times with PBS and stored overnight in PBS. In addition, they were incubated in serum free media for at least an hour before cell seeding.

4.12 Endothelial cell retention studies

To observe early time-point endothelial cell retention on three-dimensional elastin scaffolds under static and dynamic conditions, LDH assay (CytoTox® Non-radioactive

Cytotoxicity assay, Promega Corp., Madison, WI) was used. Briefly, HUVEC were trypsinized, suspended in serum free media and seeded at a concentration of 20,000 cells/cm^2 on elastin scaffolds and placed in the incubator at 37^oC, 5% CO_2 for 10, 30 minutes under static or shear conditions. Plain TCPS served as the baseline control and n= 4-5 samples were used per group. Unattached cells were washed off and cell retention was quantified by measuring the levels of LDH in the sample, as previously described. A standard curve constructed with known cell numbers was used to quantify the results obtained.

4.13 Adhesion inhibition assay

Adhesion inhibition assays were aimed at identifying the integrin receptors which play a role in cell adhesion to purified elastin scaffolds. TCPS served as the baseline control while collagen (PureCol from Inamed Biomaterials, Fremont, CA) was used as the positive control. 8 parts of chilled PureCol was mixed with 10X PBS buffer, adjusted the pH to 7.4±0.2 and allowed to gel in 96-well microplates. The gels formed were dried overnight to obtain a uniform coating of collagen on the TCPS substrate. The films were washed with distilled water to remove any precipitated salts and to rehydrate the collagen prior to cell seeding. Three distinct inhibitory antibodies were used, directed against the αv (Anti-human integrin αv Mouse mAb (272-17E6), Calbiochem- EMD, La Jolla, CA), α4 (Mouse anti human CD49d (HP2/1), Serotec, Raleigh, NC) and β1 (Mouse anti-human integrin β1 mAb (P4C10), Chemicon- Millipore, Billerica, MA) integrin receptors and a no antibody group served as the internal control (n = 4-6 per group).

The scaffolds were incubated in serum free media for at least an hour before the experiment. HUVEC suspended in serum free media were seeded on the substrates at a concentration of 20,000 cells/cm^2, since serum proteins can potentially interfere with/ enhance cellular attachment. Simultaneously, the inhibitory antibodies were also added in a ratio of 1:1 by volume. The cells were incubated with the substrate for 30, 45 and 60 minutes under static conditions in a sterile incubator at 37^oC, 5% CO_2.

At the end of the incubation period, cellular attachment in response to inhibition of specific integrin receptors was analyzed using the quantitative, colorimetric lactate dehydrogenase assay (CytoTox® Non-radioactive Cytotoxicity assay, Promega Corp., Madison, WI). Cellular attachment was expressed as relative percentage attachment, normalized to the no antibody control group for each substrate.

4.14 Statistical analysis

All statistical analysis was performed by single-factor analysis of variance (ANOVA) and the values expressed in terms of mean ± standard error of the data points within the group. $p \leq 0.05$ was considered statistically significant.

CHAPTER 5 – RESULTS AND DISCUSSION

5.1 Characterization of 3D scaffolds

5.1.1 Histology

The purity of 3D collagen and elastin scaffolds prepared was confirmed by Masson's trichrome and VVG staining. Fresh carotids were used as positive controls.

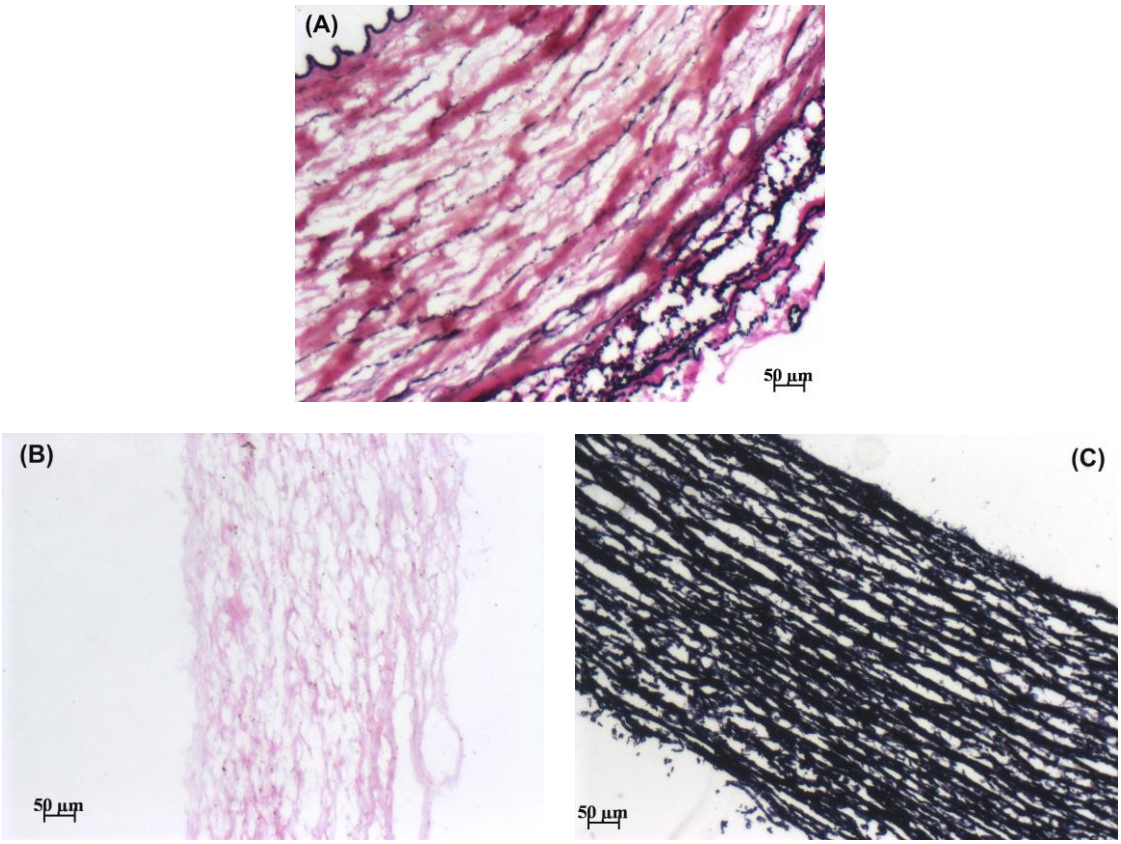

Fig 5.1: Histological sections of (A) fresh carotids, (B) collagen and (C) elastin scaffolds stained with VVG which stains collagen red-pink and elastin fibers black in color. Original image magnification – 100X.

With VVG staining (Figure 5.1), fresh arteries showed characteristically stained black elastic fibers and the collagen stained red to pink in color. The elastin scaffolds prepared by CNBr treatment showed dense black fibers confirming that purified elastin was retained with the complete removal of collagen. On the other hand, collagen scaffolds showed a light pink staining with black fibers being visibly absent.

Furthermore, alcian blue staining was performed to confirm complete removal of GAG and cellular material (Figure 5.2). It was seen that purified elastin scaffolds were completely devoid of nuclei (counterstained with nuclear red). Also, GAGs that stained blue in the fresh tissue were absent in the elastin scaffolds.

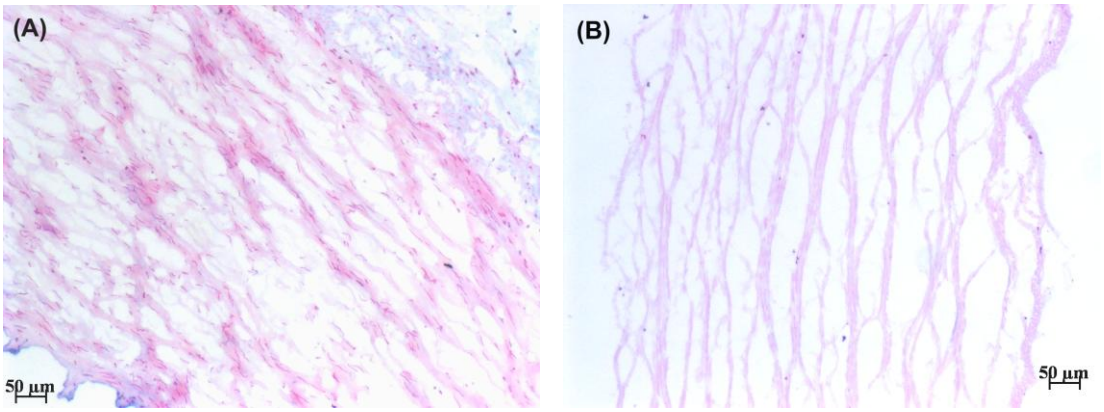

Fig 5.2: (A) Fresh carotids stained with alcian blue showing GAG stained blue and nuclei stained deep pink and (B) elastin scaffolds showing removal of GAG and cellular contents. Image original magnification – 100X.

Similarly, collagen scaffolds were characterized by Masson's trichrome staining (Figure 5.3). Pure collagen scaffolds stained green while the elastin scaffolds stained a

pale red. Fresh tissue showed typical green-stained collagenous connective tissue and muscle fibers stained red.

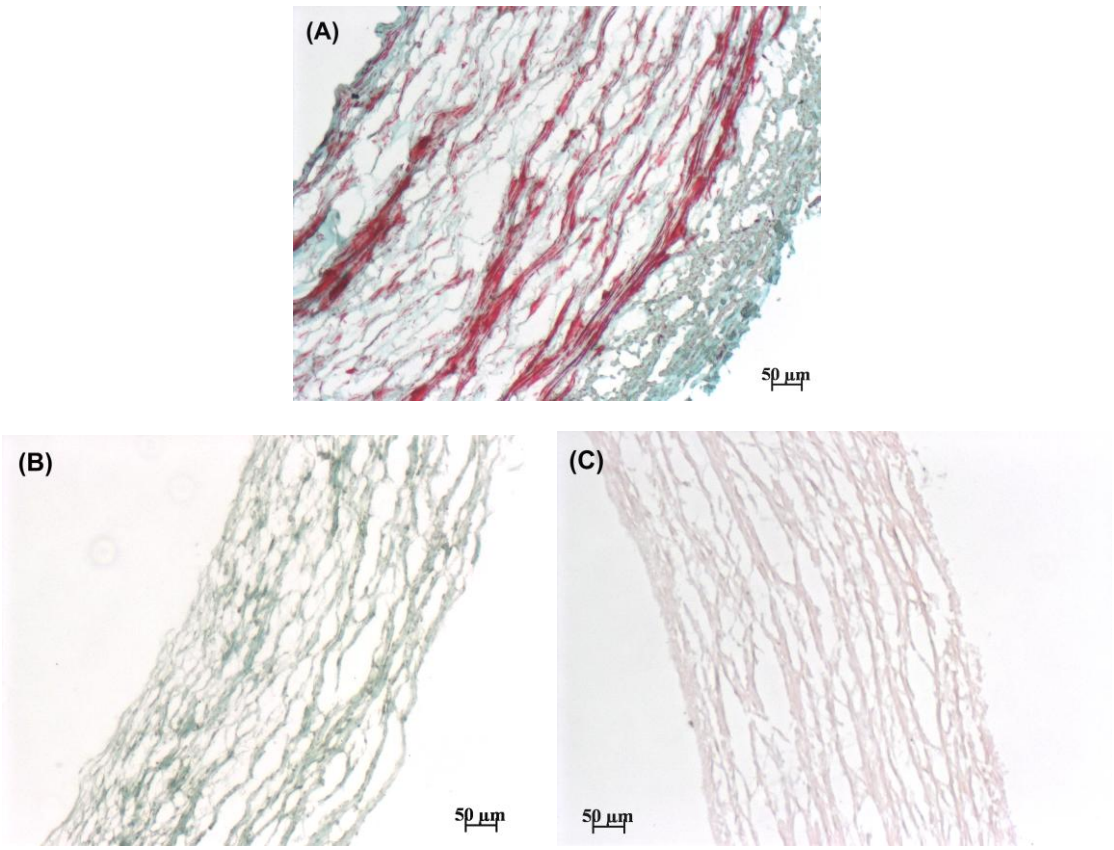

Fig 5.3: (A) Fresh carotids, (B) collagen and (C) elastin scaffolds stained with Masson's trichrome staining collagen green and muscle fibers red. Original image magnification – 100X.

5.1.2 DNA quantification assay

DNA quantification showed that approximately 97.5% of the total DNA content was removed in the elastin scaffolds, thus proving the effectiveness of the cyanogen bromide treatment (Figure 5.4).

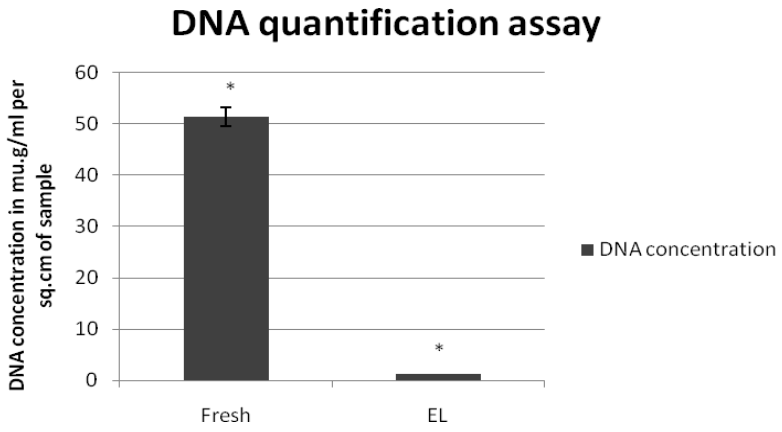

Fig 5.4: DNA quantification analysis confirms decellularization of elastin scaffolds, as compared to native artery (n=4 per group); (*) denotes $p \leq 0.05$.

However, the collagen scaffolds showed abnormal values of absorbance (even higher than the fresh artery). We assume that the various steps and agents involved in processing the collagen scaffolds could possibly interfere by absorbing light in the UV region, as histological evaluation showed preservation of purified collagen with the complete elimination of cells and other ECM components. Corroborating this view, literature reported considerable absorption of EDC (used to crosslink the collagen scaffolds) in the UV region [125]. Hence unreacted and/or unquenched residual EDC present could be the potential source for this observed anomaly.

5.2 Characterization of 2D scaffolds

5.2.1 Contact angle measurement

Contact angle measurement is a simple technique to understand surface characteristics of a material. This angle gives a measure of wettability which is a characteristic of every substrate.

Type of substrate		**Contact angle (in deg)**
Plain glass		15.66 ± 1.33
Collagen	Before hydration	38.88 ± 2.80*
	After hydration	40.71 ± 1.56*
Elastin peptides	Before hydration	37.13 ± 1.55
	After hydration	48.39 ± 1.94

Table 5.1: Air-water contact angles measured on elastin and collagen-coated glass coverslips; Values with $p \geq 0.05$ are marked with (*).

Results obtained from measuring the air-water contact angle for cleaned and uncoated glass surfaces were significantly different from the values obtained for both elastin- and collagen-coated coverslips (Table 5.1). We also measured the contact angles of the protein coated coverslips after keeping them in distilled water for 1 hour (hydration) to check if the coated protein layer remained intact. Following this, the coverslips were dried and contact angle was measured.

Glass coverslips cleaned with piranha became highly hydrophilic as expected [126, 127]. Collagen-coated glass coverslips were moderately hydrophilic, comparable to values reported in literature [128, 129]. This value did not vary significantly, before and after hydration, thereby confirming that the collagen coating was well-preserved. Glass coverslips coated with elastin peptides also showed contact angles similar to collagen-coated substrates. Unlike in the case of collagen, there was a notable increase in the contact angle of elastin-coated coverslips after treating with water. Since this value was also significantly different from that for uncoated glass, it would be safe to assume that the integrity of the coating was not compromised.

It should be noted that the elastin peptides used were a mixture of peptides of different molecular weights ranging between 1-60 kDa, containing the functional VGVAPG sequence of elastin. Hence structurally, they are very different from the native insoluble, mature, and highly hydrophobic elastin. Also, the charges present at the ends of each peptide chain can favorably interact with the hydrophilic glass substrate. Hence, we observe moderately hydrophilic values for the air-water contact angle of elastin-coated coverslips, before hydration.

Water molecules from the bulk solvent can interact with the elastin polypeptide chain and its tightly associated hydration shell, resulting in increased mobility of the chains [130]. This could lead to an increased interaction and subsequent clustering of hydrophobic residues. Also the hydration water in itself contributes to the elasticity of elastin [130, 131]. Such interactions between water molecules and the adsorbed elastin

peptides could potentially explain the observed increase in contact angle for elastin-coated substrates after the hydration step. In addition, it is also possible that small molecular weight peptide chains were washed off during hydration, leaving behind larger peptides making the substrate more hydrophobic.

5.3 Platelet studies

Platelet adhesion and activation are important events that eventually lead to biomaterial associated thrombosis. *In vitro* hemocompatibility studies hence assess platelet response to biomaterials to investigate their thrombogenicity. The behavior of platelets with an artificial surface can be highly influenced by the presence of plasma proteins and hemodynamic factors such as shear stress.

Earlier studies have shown that elimination of plasma proteins or use of suspension media other than plasma significantly alters platelet response to test materials [132, 133]. Similarly, it is important to include shear stress while evaluating hemocompatibility of materials since thrombus formation *in vivo* occurs under flow conditions. The mechanism of shear-mediated platelet response is primarily transduced by means of plasma proteins [134]. Therefore both plasma proteins and shear must be incorporated to truly evaluate the performance of blood-contacting biomaterials. In the present study, we attempt to delineate the effects of these two major factors in influencing the behavior of platelets in response to purified elastin and collagen scaffolds, in an *in vitro* testing system.

Different systems such as cone and plate rheometer, parallel plate flow systems, orbital shaker etc. have been used to test materials under shear. While parallel plate chambers simulate laminar shear conditions, it must be noted that flow conditions under pathological conditions or at anastomotic sites are often disturbed and non-laminar. Hence in this study we chose the orbital shaker system to study the influence of shear on platelet interactions with the substrate [135, 136]. This setup is inexpensive, does not require high volumes of platelet suspensions (as opposed to the parallel plate flow chamber) and models disturbed flow patterns that the biomaterial will be exposed to immediately post implantation.

5.3.1Quantification of platelet attachment (LDH assay) and morphological analysis of platelet activation (SEM)

For the platelet attachment studies, sigmacote-treated glass coverslips were used as negative control and showed negligible amounts of platelet attachment both with platelet rich plasma (PRP) and washed plateletsuspension (WP). All statistical analysis were performed between collagen and elastin samples (that were tested under the same mechanical conditions, with the same platelet suspension and for the same duration) and $p \leq 0.05$ was considered significant.

All SEM studies were performed at the end of a 60 minute incubation of the substrate with the platelet suspension, since we were interested in studying platelet response to the scaffolds after allowing them to interact for the longest test period.

5.3.1.1 Platelet attachment on 3D elastin and collagen scaffolds

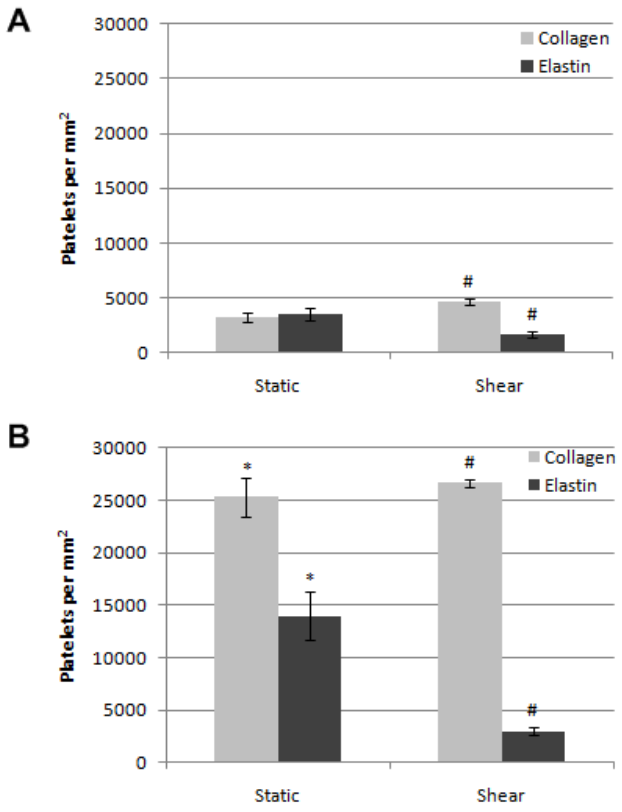

Fig 5.5: Platelet attachment with PRP on elastin and collagen 3D scaffolds after (A) 15 minute and (B) 60 minute incubation showing lower platelet attachment on elastin scaffolds (* and # represent $p \leq 0.05$).

As presented in Figure 5.5, with PRP there was no significant difference in platelet attachment between elastin and collagen at 15 minutes under static conditions. But after 60 minutes incubation, the difference was marked. When the scaffolds were tested under shear, elastin supported much less adhesion as compared to collagen, both after 15 and 60 minutes incubation indicating its superior platelet-resistant properties.

While platelet attachment levels did not vary between static and shear conditions for collagen, elastin samples tested under shear showed a much reduced number of attached platelets. This could be explained by the reduced interaction time for platelets with the substrate under dynamic testing conditions when compared to a static testing system. Since shear is known to cause conformational changes of platelet receptors leading to platelet binding to collagen [134], the influence was higher in collagen while elastin's lack of receptors for platelet binding caused no increase in platelet binding under shear.

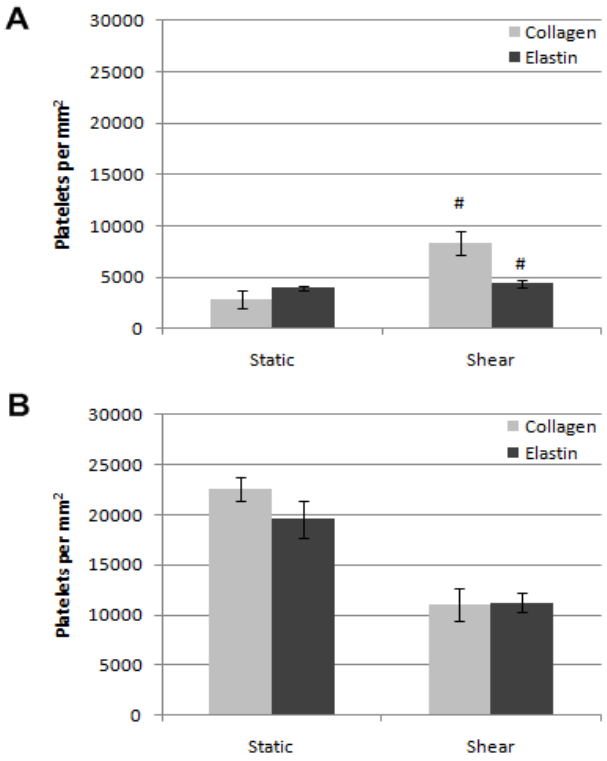

Fig 5.6: Platelet attachment with WP on elastin and collagen 3D scaffolds after (A) 15 minute and (B) 60 minute incubation showing similar platelet behavior on collagen and elastin when plasma proteins are removed. (# represents $p \leq 0.05$).

In the absence of plasma proteins, there was almost no difference in the behavior of elastin and collagen scaffolds with respect to platelet attachment (Figure 5.6). Elastin scaffolds showed a higher number of adhered platelets with WP than with PRP, both under static and dynamic conditions. This could be attributed to the absence of plasma proteins which would initially adsorb on the elastin surface. Since elastin probably does not cause any structural rearrangements in these proteins, platelets interacted poorly with the substrate when plasma proteins were present. However when this component was removed, non-specific weak interactions could be possible with the underlying elastin substrate.

For collagen scaffolds, the number of platelets attached with WP under static conditions was comparable to the number observed with PRP. On the other hand, initially shear seemed to increase platelet attachment when tested with WP, but after 60 minutes this effect was not evident. Platelet-collagen interactions are mediated by a variety of receptors, both directly and through other plasma proteins such as vWF and Fg [137-139]. Hence, even in the absence of plasma proteins platelet attachment levels were not greatly reduced.

5.3.1.2 Platelet activation on 3D elastin and collagen scaffolds

SEM images of purified collagen and elastin 3D scaffolds clearly showed the differences in surface topography between the two scaffolds, as seen in Figure 5.7. While the fibers on elastin scaffolds were thicker and clearly defined, the collagen scaffold

looked very irregular at the outset. At higher magnifications, thinner and more uneven fibers could be identified.

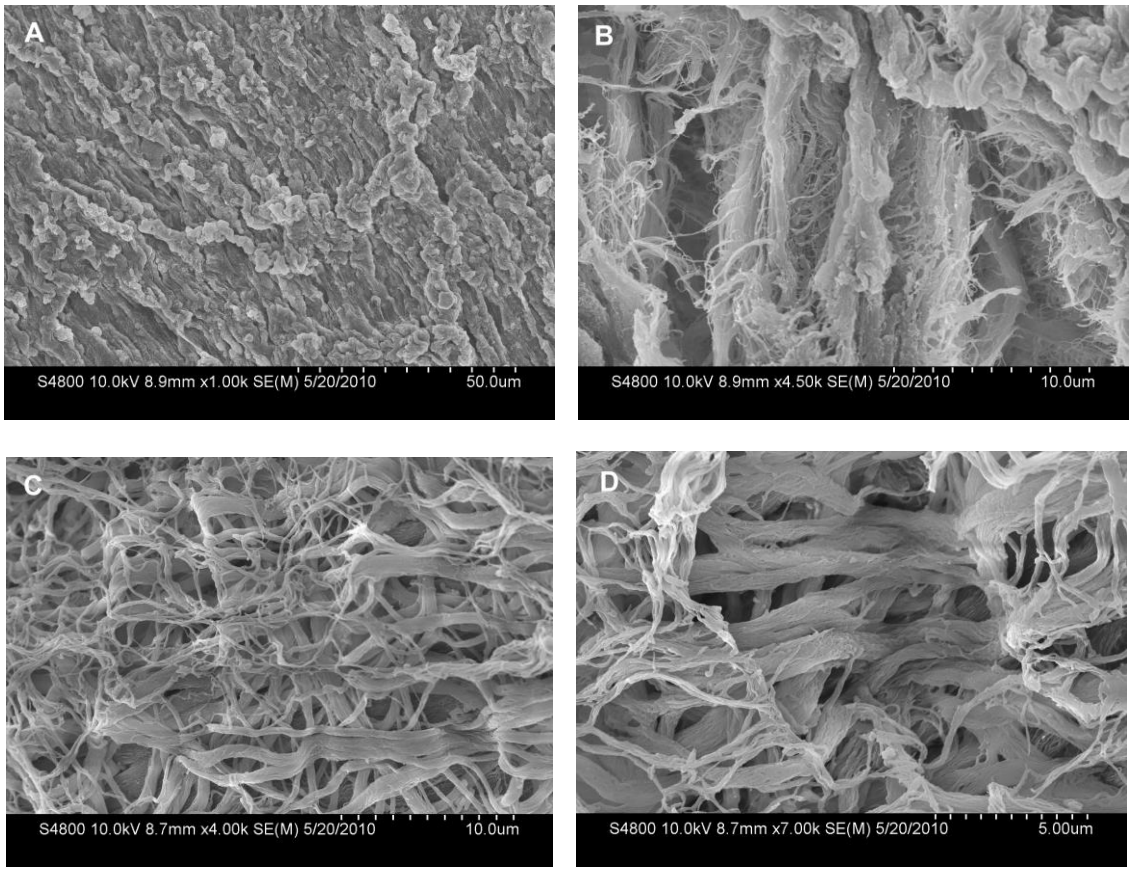

Fig 5.7: SEM micrographs showing plain purified 3D collagen scaffolds (A & B) and elastin scaffolds (C & D).

Similar to the results obtained with quantitative platelet attachment studies, SEM also revealed the superior platelet resistance of elastin scaffolds with PRP, both under static and shear conditions (Figure 5.8). In fact, under shear very few platelets could be spotted, and only at very high magnifications. Also, the attached platelets were rounded or dendritic with very limited pesudopodial extensions.

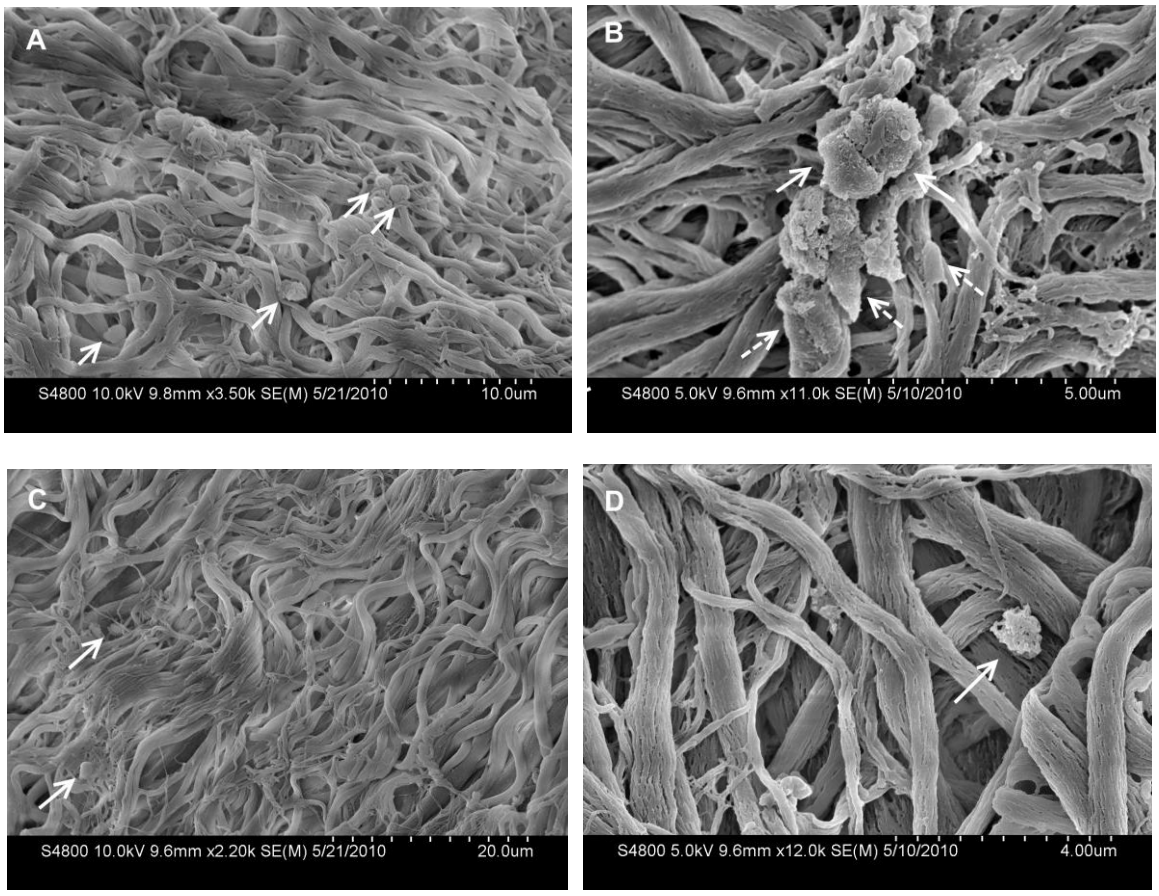

Fig 5.8: SEM images of platelets attached to 3D elastin scaffolds with PRP under (A&B) static and (C&D)shear conditions. Solid arrows indicate rounded platelets and dashed arrows point to dendritic or spread dendritic morphologies.

On the contrary, collagen scaffolds showed a noticeably higher occurance of attached and activated platelets. Due to irregularities with surface topography, the adhered platelets could be discerned only at moderately higher magnifications. It can be seen from Figure 5.9 that there was no observable difference in the surface coverage of the activated platelets between samples tested under static and dynamic conditions. Overall, based on morphology most of the platelets were spread if not fully flattened.

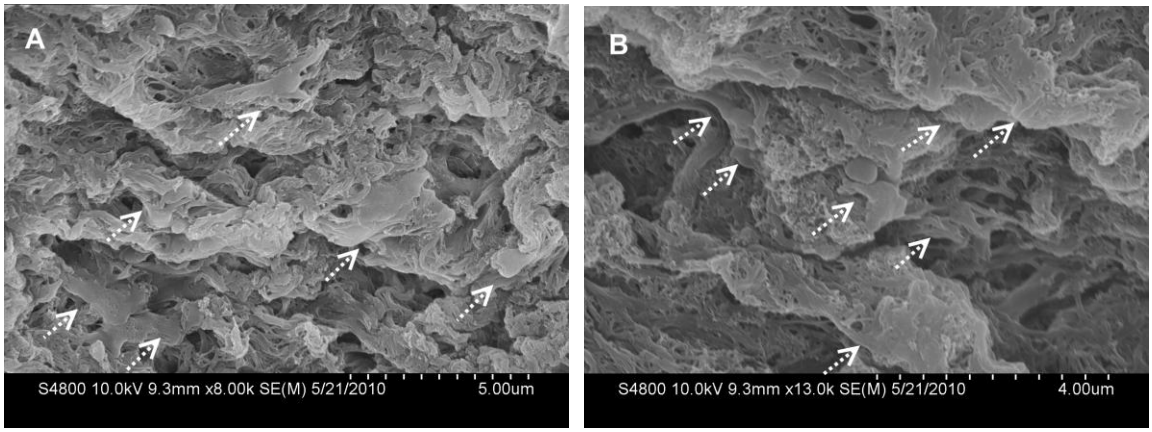

Fig 5.9: SEM images of attached and activated platelets on 3D collagen scaffolds with PRP under (A) static and (B) shear. Dotted arrow marks denote spread/fully flattened platelets.

When the scaffolds were incubated with the washed platelet suspension which does not contain plasma proteins, both elastin and collgen showed a higher degree of activated platelets on their surface.

Elastin scaffolds showed both a higher surface coverage and a higher proportion of spread and fully flattened platelets under static conditions, whereas samples tested under shear showed fewer platelets which were morphologically rounded, dendritic or spread-dendritic (Figure 5.10).

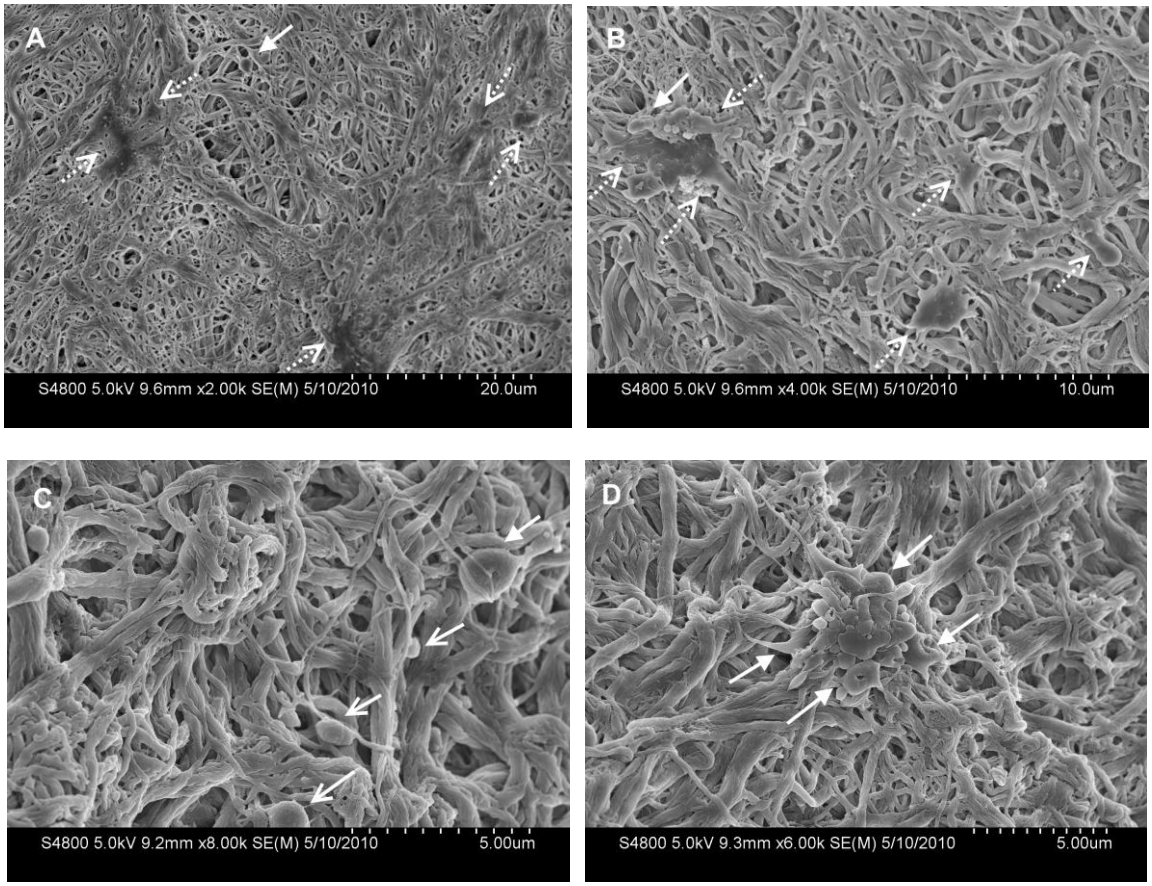

Fig 5.10: SEM images showing platelet activation on 3D elastin scaffolds with WP under (A&B) static and (C&D)shear conditions. Plain arrows ($\rightarrow$) indicate rounded platelets, filled arrows (➔) show dendritic morphology and dotted arrows denote spread/flat platelets.

Collagen scaffolds treated with washed platelets showed a higher number of platelets covering the surface under static conditions than under shear (Figure 5.11). However, morphologically the platelets under both states were spread with extended pseudopodia and some were also found to be completely flat.

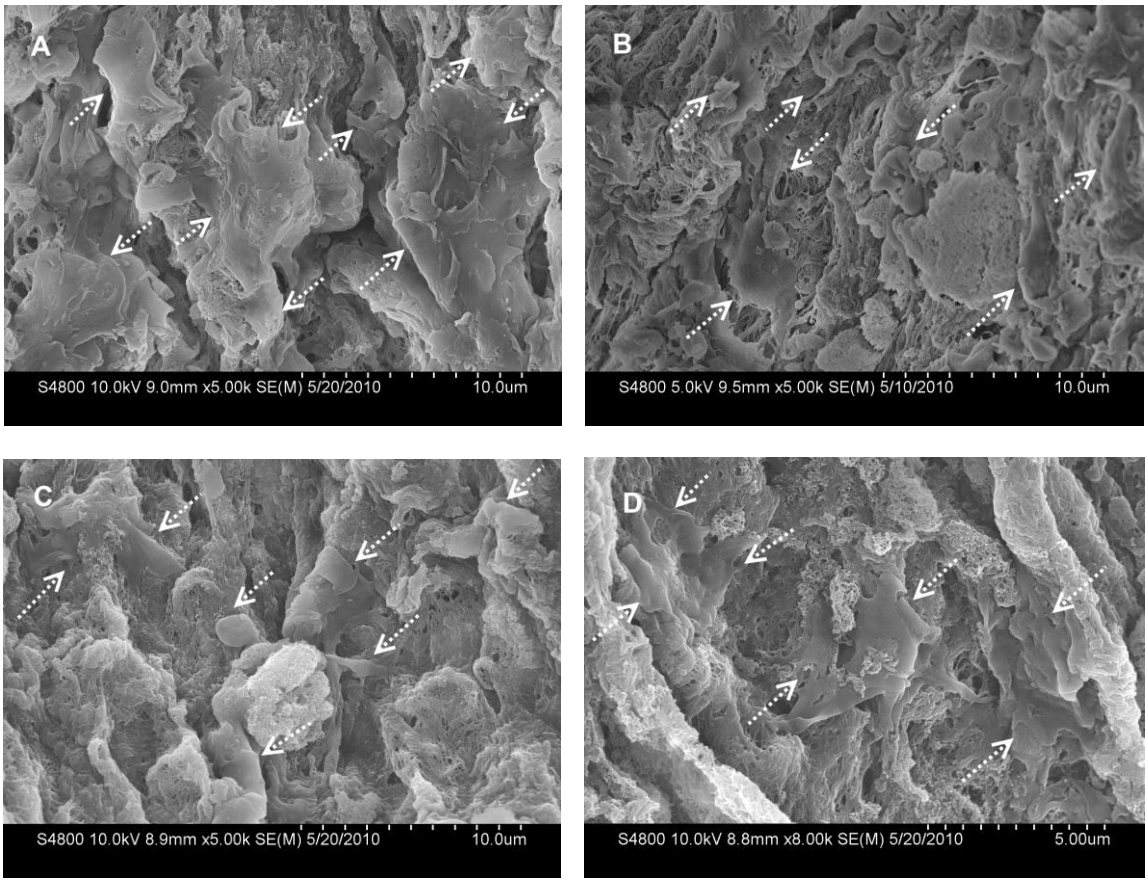

Fig 5.11: SEM images of activated platelets on 3D collagen scaffolds with WP in the absence (A&B) and presence (C&D) of shear. Dotted arrow marks point to fully activated (spead and flat) platelets.

5.3.1.3 Platelet attachment 2D elastin and collagen scaffolds

We further tested platelet binding on 2D scaffolds (thin films) to find out if the data obtained in 3D scaffolds was due to different porosities of collagen and elastin scaffolds. Platelet attachment with PRP, on elastin and collagen-coated 2D converslips also showed similar results as 3D scaffolds, with a significant increase in the number of

platelets attached to collagen than elastin, both under static and shear conditions (Figure 5.12).

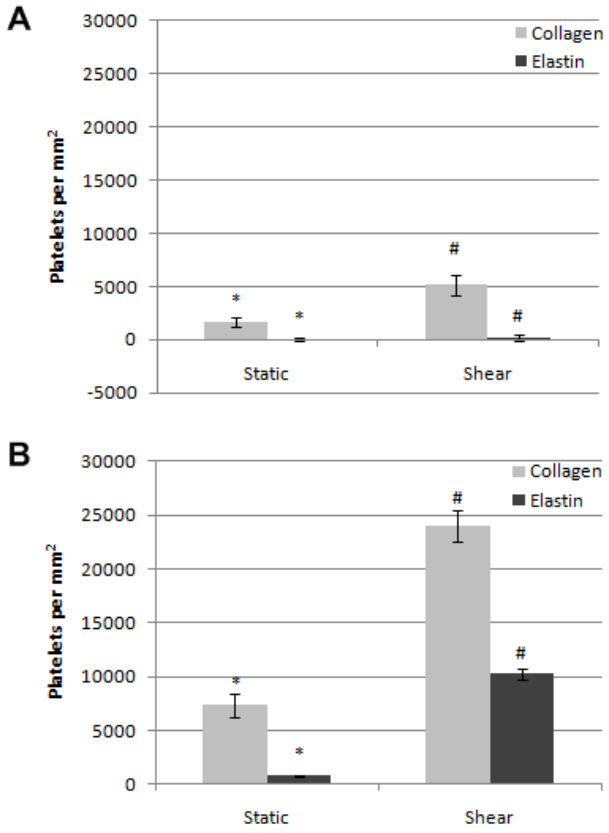

Fig 5.12: Platelet attachment with PRP on 2D elastin and collagen coated coverslips after (A) 15 minute and (B) 60 minute incubation. Elastin showed significantly lower platelet attachment than collagen (* and # represent $p \leq 0.05$).

Once again, this trend was not observed with washed platelet suspension, especially under shear (Figure 5.13). It is also important to note that in the presence of shear, the number of platelets attached to both the scaffolds was higher with washed

platelets than with PRP (Figure 5.12 and 5.13). Thus plasma proteins present in PRP are actually protecting both surfaces from platelet binding.

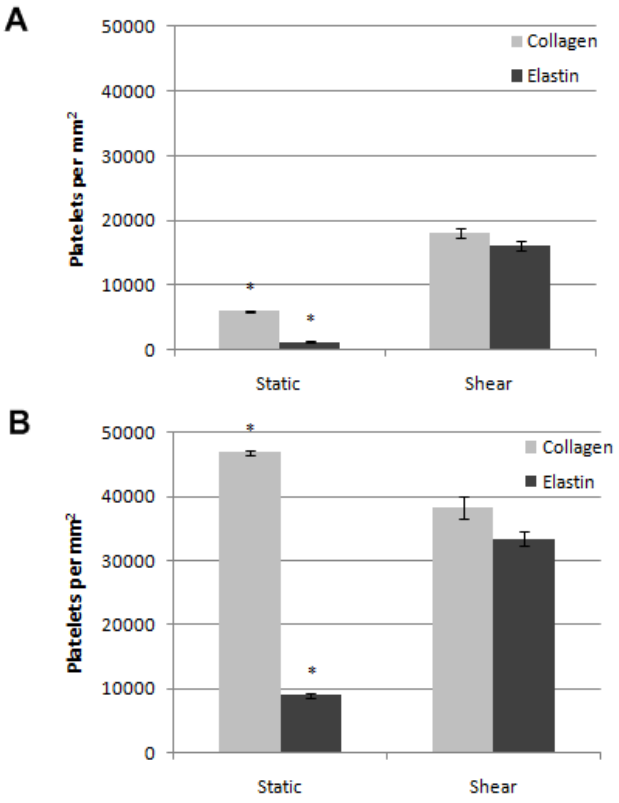

Fig 5.13: Platelet attachment with WP on 2D elastin and collagen coated coverslips after (A) 15 minute and (B) 60 minute incubation showing increase in platelet attachment to elastin, in response to the absence of plasma proteins (* and # represent $p \leq 0.05$).

With both collagen and elastin-coated 2D surfaces, platelet attachment under shear was higher (barring collagen-WP-60 minutes) than what was observed under static conditions. This could be the effect of excessive deformation caused by subjecting the platelets to shear.

Interestingly when tested with washed platelets, it was observed that quantitatively the number of platelets attached on 2D collagen surfaces was higher than 3D scaffolds (Figures 5.6 and 5.13) both under static and shear conditions. On the other hand, similar trend was seen only for those elastin samples that were maintained under shear. This could potentially be due to easier accessibility of sites for platelet aggregation as topographical features like pores are absent in 2D scaffolds. Minelli et al have similarly observed that washed platelets preferably attach more to surfaces with minimal surface features and height [140].

5.3.1.4 Platelet activation on 2D elastin and collagen scaffolds

In the case of 2D scaffolds, elastin-coated coverslips showed almost no platelet attachment with PRP under static conditions unlike collagen, where a few platelet aggregates could be found as shown in Figure 5.14.

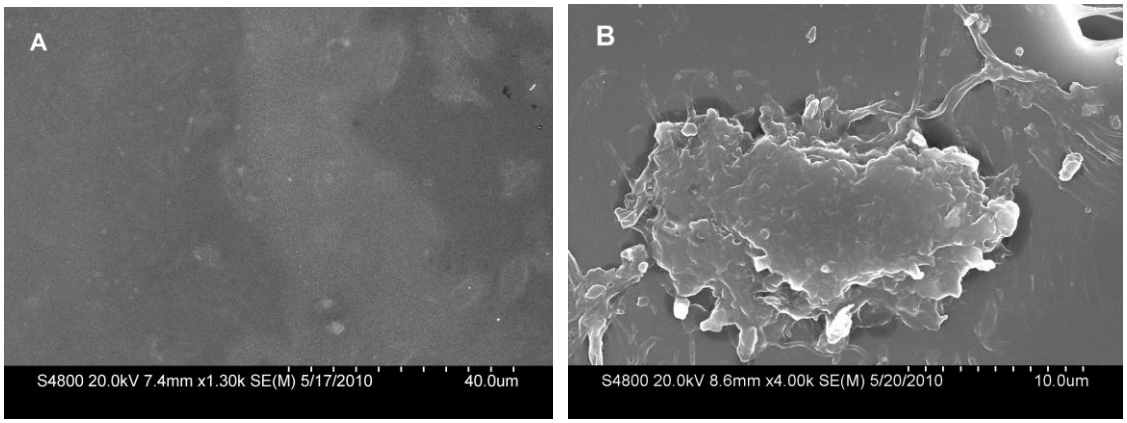

Fig 5.14: SEM micrographs of 2D elastin (A) and collagen (B) coated coverslips treated with PRP under static conditions showing platelet aggregates only on collagen.

However, no SEM images could be obtained for both elastin and collagen 2D samples subjected to shear with PRP in spite of observing results with the platelet attachment assay (Figure 5.7). This could probably be due to the loosely adhered platelets getting dislodged during the several washing steps involved in SEM sample preparation and/or the general poor interaction of PRP with protein-coated glass coverslips as compared to TCPS surface over which proteins were coated for the platelet attachment assay.

With WP, 2D scaffolds showed a significantly higher amount of platelet adhesion and activation. Elastin-coated surfaces showed a higher number of platelets, with most of them being dendritic or spread-dendritic under static conditions while under shear more clumps with spread and fully flattened platelets could be found (Figure 5.15).

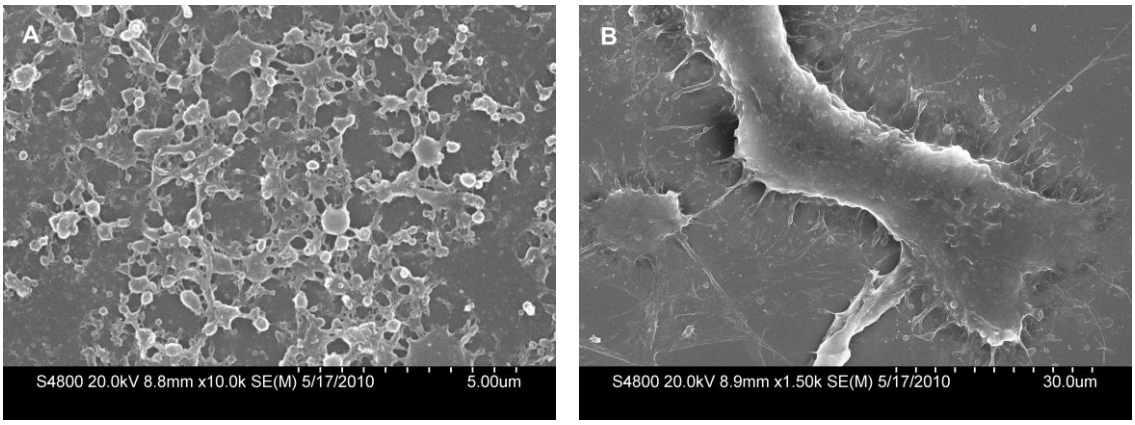

Fig 5.15: SEM images of platelet attachment and activation on elastin-coated coverslips with WP under (A) static condition showing dendritic and spread dentritic platelets, and (B) dynamic condition showing platelet spreading.

In contrast, collagen-coated coverslips were completely covered with fully flattened and a few spread platelets under static conditions (Figure 5.16). Samples incubated under shear showed a comparatively lower surface coverage with a few patches of the underlying collagen being seen. The morphology of the platelets was similar to that of scaffolds maintained under static conditions.

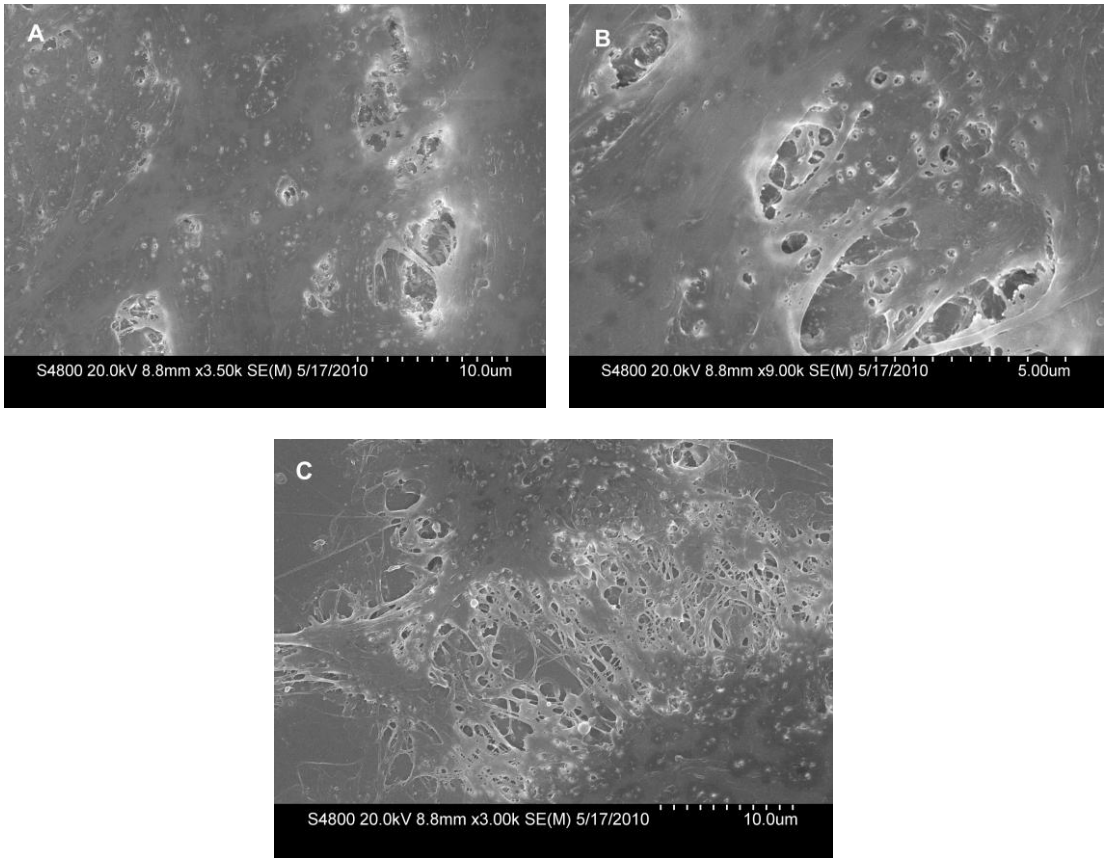

Fig 5.16: SEM micrographs of activated and spread platelets found on 2D collagen surfaces with WP under (A&B) static and (C) shear.

The following table (Table 5.2) summarizes the results observed with SEM, in terms of morphological grading of platelets adhered to both 3D and 2D collagen and elastin scaffolds. Platelets were assigned grades from 1 to 5 in the increasing order of

activation with rounded platelets denoted as 1, dendritic platelets as 2, spread dendritic platelets as 3, spread platelets as 4 and fully spread/flattened platelets as 5.

Substrate and testing condition			**Morphological grading**
Elastin 3D	PRP	Static	1,2
		Shear	1
	WP	Static	4,5
		Shear	3,4
Collagen 3D	PRP	Static	4,5
		Shear	4
	WP	Static	5
		Shear	5
Elastin 2D	PRP	Static	0
	WP	Static	2,3
		Shear	5
Collagen 2D	PRP	Static	4
	WP	Static	5
		Shear	4,5

Table 5.2: Subjective gradation based on morphology of adhered platelets on 2D and 3D elastin and collagen scaffolds observed with SEM.

Taken together, it can be concluded from the platelet studies that in the presence of plasma proteins, which more closely resembles the conditions *in vivo*, elastin is much more resistant to platelet adhesion and activation both under static conditions and under shear.

As expected, the 3D porous elastin scaffolds presenting a larger surface area showed higher number of platelets attached than elastin-coated 2D coverslips. Also, under shear fewer platelets were found on elastin scaffolds. When the plasma proteins were removed, the demarcation between collagen and elastin scaffolds was not clear, especially under shear when both elastin and collagen showed similar platelet adhesion. However, with respect to platelet activation, elastin scaffolds showed only isolated aggregates even with washed platelets under shear, unlike the highly thrombogenic collagen substrates.

5.4 Plasma recalcification time

Biomaterial contact is known to cause activation of Hageman factor (Factor XII) and set off the intrinsic pathway of coagulation. It involves a cascade of reactions in which calcium acts as a cofactor, ultimately resulting in the formation of a fibrin clot. Since this process is dependent on the material's surface properties, the dynamics of plasma clotting reflecting this activation can be used to assess the hemocompatibility of different biomaterials [141]. Glass which has a negatively charged, hydrophilic surface has shown to strongly support contact activation and hence was used to compare the values obtained for the test substrates. Recalcified plasma was added to the test samples

and the study was conducted for 20 minutes, which extends well beyond the time required for contact phase activation of normal plasma [142].

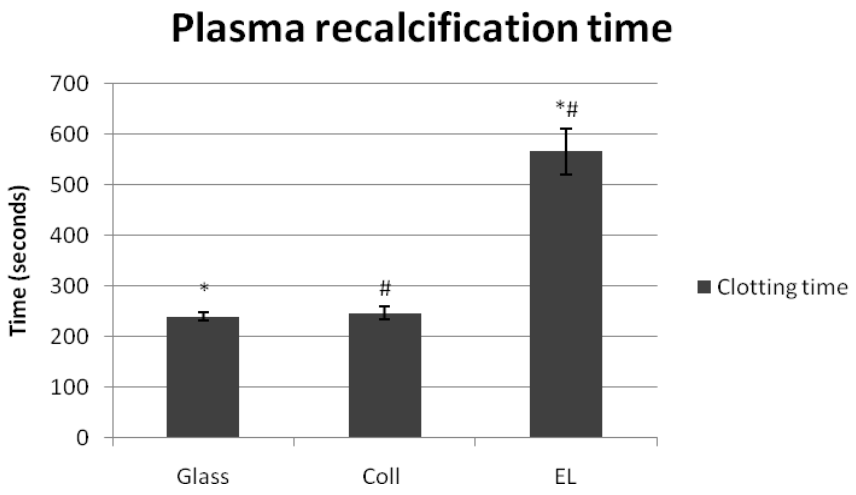

Fig 5.17 : Clotting time of recalcified plasma measured on glass, collagen and elastin substrates (* and # indicate $p \leq 0.05$).

Results obtained from the plasma recalcification analysis again proved that collagen possessed much higher procoagulant activity than elastin (Figure 5.17). With elastin, clotting time of approximately 567 ± 45 s was observed. Both collagen and glass showed significantly lesser times of about 246 ± 13 s and 240 ± 9 s respectively. Native collagen is documented to activate Hageman factor and the observed values agree with reported clotting times [114, 143]. We also checked the recalcification time on plain microcentrifuge tubes in which the reaction was carried out with the 3D scaffolds. None of the tubes (n = 5) showed coagulation within the test period of 1200 s.

5.5 Circular dichroism

Fibrinogen plays an important role in blood-biomaterial interactions. It is one of the major constituents of the adsorbed protein layer on a biomaterial surface, owing to its high plasma concentration. Additionally, Fg adsorption and subsequent deformation largely influences platelet response to a foreign material [144]. From the platelet studies, it was evident that the presence of plasma proteins was essential to fully understand biomaterial hemocompatibility and neglecting this factor could greatly modify the platelet response. Hence we chose to study the adsorption of fibrinogen, one of the main players in this process, on elastin and collagen-coated substrates.

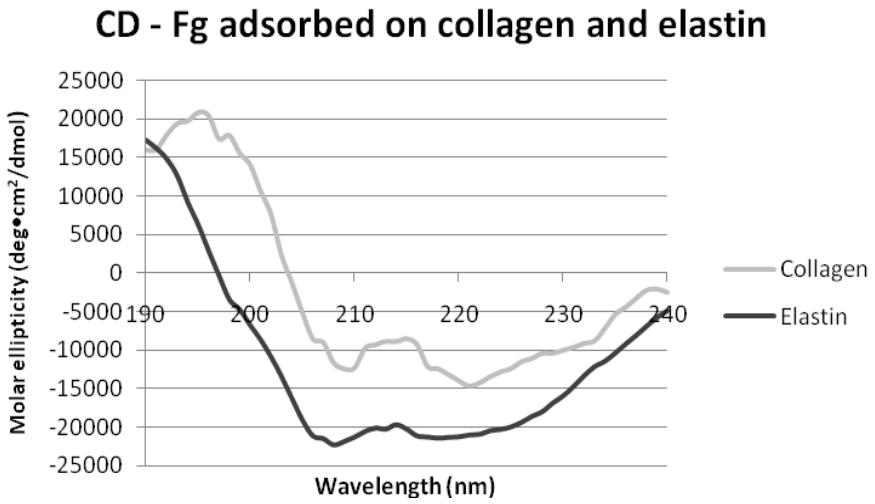

Fig 5.18: Representative CD spectra of fibrinogen adsorbed on to elastin- and collagen-coated quartz slides.

Representative spectra of molar ellipticity Vs. wavelength (Figure 5.18) seemed to indicate differences in the negative peaks found at around 208 and 222 nm (important

in determining α-helical structures) for Fg adsorbed between collagen and elastin-coated surfaces.

However, the percentage of secondary structural components, α-helix and β-sheet as evaluated by the SELCON software did not show any significant difference between the elastin- and collagen-coated quartz surfaces as compared to the corresponding values for native fibrinogen in solution (Figure 5.19) .

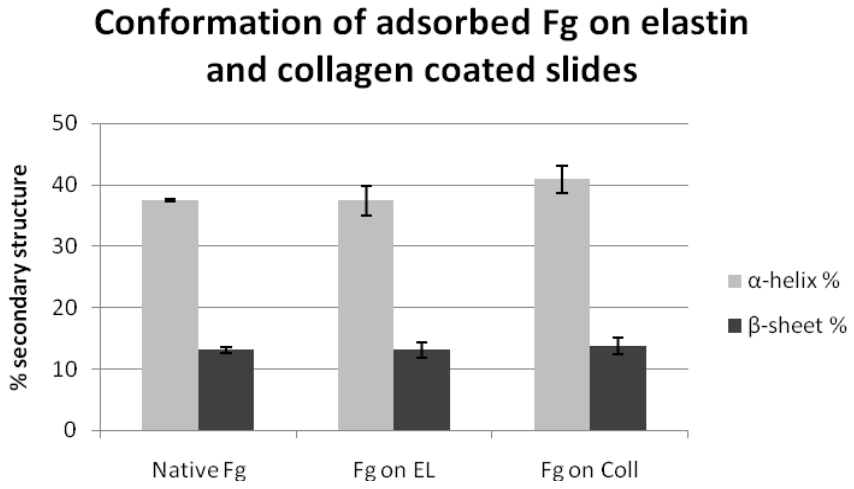

Fig 5.19: Percentage composition of secondary structures of adsorbed Fg on protein-coated substrates, compared to native Fg conformation. $p \leq 0.05$ was considered statistically significant.

This apparent similarity in behavior for collagen and elastin might in fact be due to the high concentration of protein used, which could potentially saturate the surface, thereby masking any differences of the actual surface-contacting protein layer. A higher degree of unfolding and denaturation was expected on the collagen-coated substrates,

since plasma protein unfolding is believed to present binding sites for other cellular components, ultimately contributing to the higher thrombogenicity of collagen as observed in platelet studies. However, the relative significance of direct collagen-fibrinogen interactions is unclear as other plasma proteins such as vWF and fibronectin are primarily thought to interact with collagen [137, 145-147]. This could possibly explain the absence of any major changes in the observed values for the percentage composition of secondary structures of adsorbed Fg on collagen-coated quartz slides.

One of the inherent limitations of working with protein-based substrates is the inability to follow rigorous cleaning procedures to ensure absolute purity of the substrate, as in the case of synthetic materials. Hence the possibility of interference due to surface-bound impurities while taking background spectra measurements cannot be eliminated.

Type of substrate	**Surface coverage of Fg adsorbed ($\mu g/cm^2$)**
Elastin-coated quartz	0.8175 ± 0.059
Collagen-coated quartz	0.4673 ± 0.030

Table 5.3: Amount of fibrinogen adsorbed in $\mu g/cm^2$ on elastin and collagen-coated quartz slides obtained from CD spectropolarimetry.

Surface coverage was estimated based on the modified equation for calculating molar ellipticity from adsorbed protein concentration. Elastin-coated quartz surfaces

showed a marginally higher (albeit statistically significant) value for surface coverage than collagen-coated surfaces, as shown in Table 5.3. Interestingly, it can be noted from contact angle measurements that after incubation with water, elastin-coated coverslips were slightly more hydrophobic than collagen-coated substrates. For a given bulk protein concentration, the amount of adsorbed protein (represented here by surface coverage) increases with increasing hydrophobicity [148]. Also, surface coverage is inversely related to the extent of protein denaturation. The denser the surface packing of the adsorbed protein, the more they are restricted from structural rearrangement and denaturation [148]. Therefore, the lesser surface coverage of adsorbed Fg on collagen-coated might in turn provide a greater opportunity for the protein to spread out.

5.6 Endothelial cell retention

Retention of seeded endothelial cells is critical for the performance of any cardiovascular biomaterial, especially to prevent unfavorable reactions with the host cells and plasma components immediately post-exposure. In this study, we investigated the ability of the purified 3D elastin scaffolds to support endothelial retention by itself, in the absence of serum proteins *in vitro* (since the amount of serum used for *in vitro* culture is extremely high when compared to physiological levels).

It was observed that endothelial cell retention was comparable to TCPS at the end of the 10 minute incubation period, both under static and shear conditions. Under static conditions, the 3D elastin scaffolds showed good cellular retention, even after 30 minutes, in the absence of serum proteins. However under shear, elastin was not capable

of retaining the majority of the cells when the incubation was extended to 30 minutes (Figure 5.20).

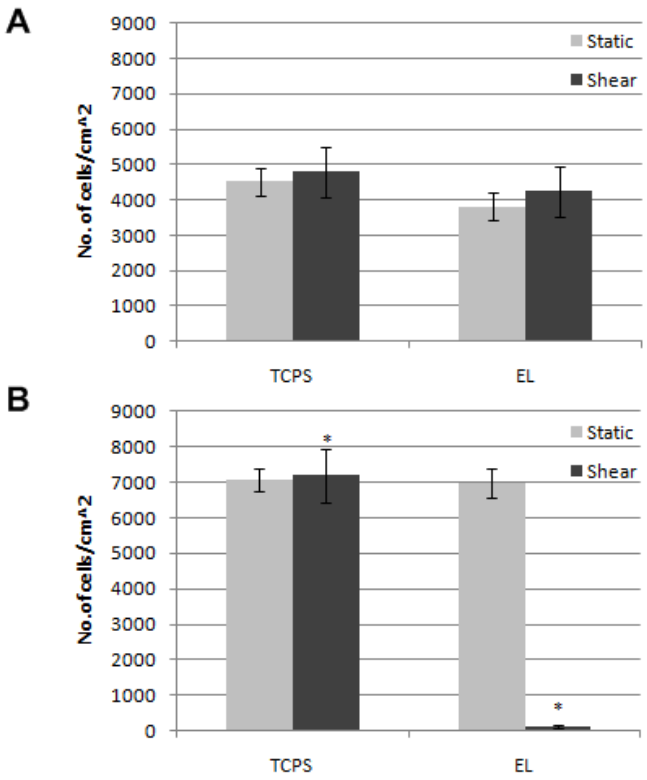

Fig 5.20: Early time-point endothelial cell retention on elastin scaffolds under static and shear conditions after (A) 10 minutes and (B) 30 minutes of cell seeding (* represents $p \leq 0.05$).

5.7 Adhesion inhibiton assay

In order to improve the poor retention of endothelial cells on elastin scaffolds under shear, it is important to understand the process of adhesion and the contribution of specific receptors. This knowledge would help in improving cellular retention by

encouraging the activation of these receptors or stimulating their upregulation through appropriate molecular signals [149-152].

Integrins are the major class of adhesion molecules involved in cell-ECM interactions. They are heterodimeric receptors consisting of an α and a β subunit and require divalent cations for their function. As our test material elastin, was a purified ECM component, we chose to study the role of integrin receptors in mediating adhesion of the seeded endothelial cells to the scaffold.

In the present study, three specific integrin subunits αv, α4 and β1 were assayed since their involvement has been implicated in endothelial adhesion to tropoelastin and other related subendothelial elastic fibre-associated molecules [153-157]. As we performed an adhesion inhibiton assay, the relative contribution of an individual receptor was deciphered by the extent of reduction in cellular retention upon its inhibition.

Our results showed that both αv and β1 inhibition resulted in a distinct reduction in cellular retention of up to 25% with elastin, but α4 blocking did not have any effect after 30 minutes of incubation (Figure 5.21). By 45 minutes, the percentage retention had gone over 90% in all the groups, while there was no difference between the integrin-inhibited and the no antibody control group at the end of 60 minutes incubation.

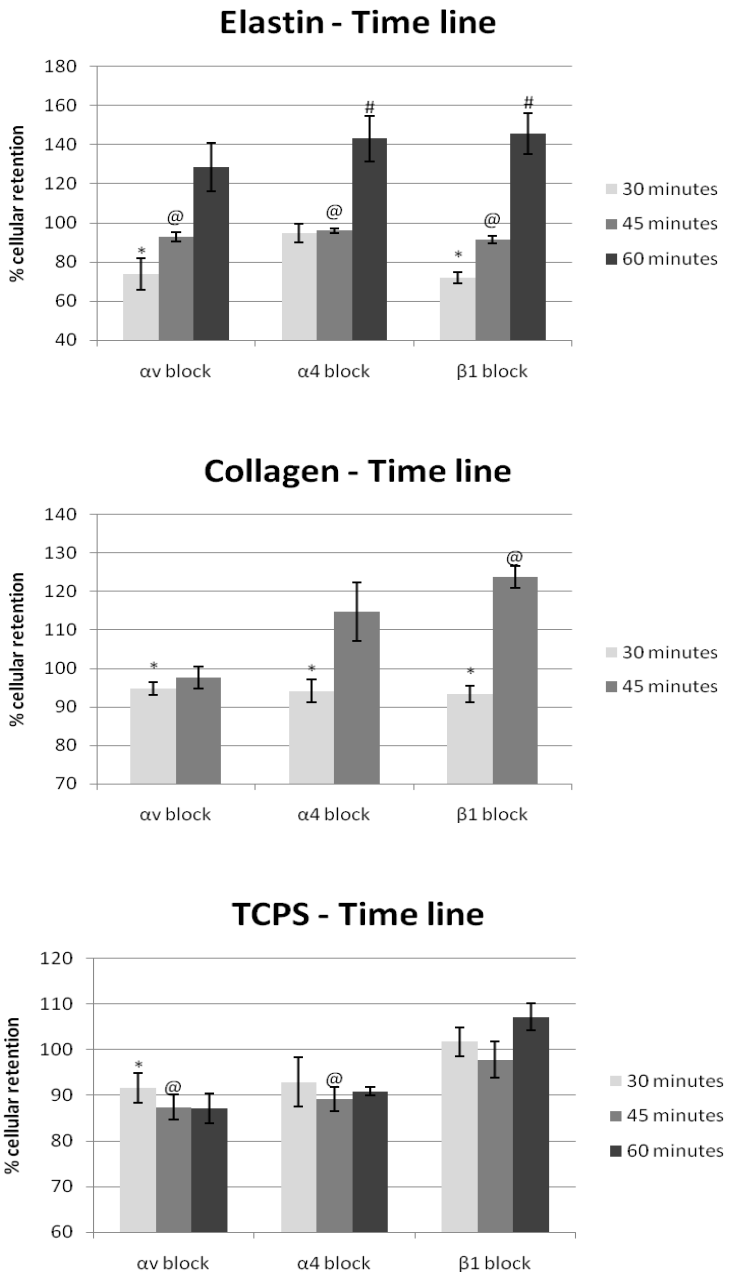

Fig 5.21: Adhesion inhibition assay on elastin, collagen and TCPS to understand involvement of integrin receptors in early adhesion of endothelial cells. All statistical analysis performed between sample group and no antibody control with $p \leq 0.05$ being considered statistically signigicant (denoted by *,@ and #).

With the positive control, collagen, all the three integrin receptors appeared to contribute to adhesion. However, the decrease was not pronounced, approximately 6-7% (Figure 5.21). This could point to the role played by multiple integrin receptors in mediating endothelial adhesion to collagen substrates. Hence, blocking of one specific receptor did not significantly alter or decrease cellular retention. At the end of the 45 minute incubation period, none of the blocking antibodies exercised any effect in inhibiting cell attachment. Predominantly α2β1, α1β1, α3β1 integrin receptors are said to be involved in interactions with collagen [158-161]. However, in our study no stark effect was observed by inhibiting the β1 subunit.

On the other hand with TCPS which was the baseline control, αv and α4 blocking decreased cellular attachment by 7-8% after 30 minute incubation and this effect continued even after 45 minutes. But by 60 minutes, the antibodies failed to produce any statistically significant reduction in endothelial adhesion. In this case, β1 blocking was not successful probably since other β receptors could be involved in attachment to TCPS.

The general trend of cell attachment observed with this adhesion inhibition assay with simultaneous introduction of cells and the blocking antibodies could be explained as follows (Figure 5.22).

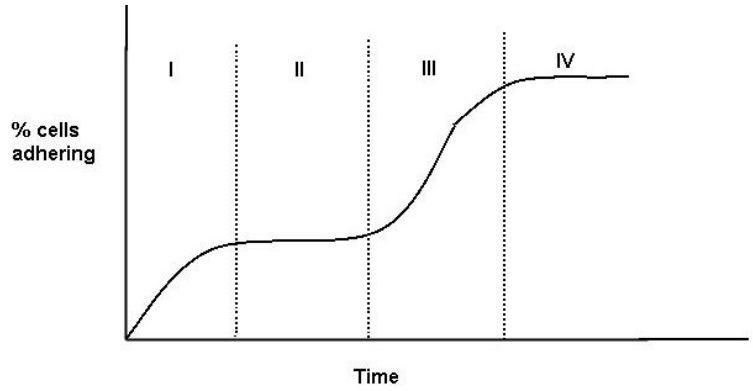

Fig 5.22: Possible trend of cell attachment with the adhesion inhibition assay to study integrin-mediated early endothelial adhesion.

Phase 1 represents the initial domination of cells attaching to the substrate, followed by Phase 2 wherein an equilibrium is reached between the cells trying to attach to the underlying substrate and the blocking antibodies which interfere with this adhesion. Phase 3 denotes the modulation of the cells to the new environment by compensating for the blocked receptors through facilitating attachment through different integrin receptors. Finally phase 4 or saturation is reached once the adhesion levels reach on par with the no antibody control.

Based on this hypothesis, we could conclude that collagen was the most conducive substrate for endothelial attachment via various integrin receptors. Hence by 30 minutes, it was likely to be in phase 3 and by 45 minutes the final phase of saturation had been attained. With elastin, at the end of 30 minutes, there was a pronounced effect of the blocking antibodies αv and β1, probably signifying phase 2. As time progressed, at 45 minutes the percentage of cellular retention approached 100% possibly indicating the onset of phase 3 and by 60 minutes saturation was reached. On the other hand, with the

baseline control – TCPS, blocking antibodies against αv and α4 integrins had a minimal effect in inhibiting cell attachment. However, this effect was consistently seen after 30 and 45 minutes incubation. Hence, it can be assumed that with TCPS, saturation (phase 4) was reached over 45 minutes. Other integrin receptors potentially mediating adhesion to TCPS could be investigated to provide more insights into the dynamics of early endothelial attachment, as the receptors being studied did not seem to influence attachment significantly.

Overall, with respect to early adhesion of endothelial cells to elastin scaffolds, integrin receptors αv and β1 seem to contribute considerably. It should also be noted that the adopted model of simultaneous interaction of the endothelial cells and inhibitory antibodies with the test substrate does not guarantee absolute elimination of the effect of the subunit being blocked, since some degree of initial attachment might occur even before the blocking antibodies bind to the endothelial cell surface integrin receptors. Taking this into consideration, the actual influence of the specific integrin subunits could be more pronounced than what was observed in the current study.

CHAPTER 6 – CONCLUSIONS AND RECOMMENDATIONS

Hemocompatibility of purified elastin scaffolds was evaluated *in vitro* by studying platelet response to 2D and 3D scaffolds. Additionally, the influence of important factors such as presence of plasma proteins and shear was investigated. In general, elastin scaffolds showed a lower tendency to support platelet interactions when compared to equivalent protein-based scaffolds composed of collagen. Especially in a physiologically relevant milieu, in the presence of plasma proteins and shear, elastin exhibited superior platelet resistance and hemocompatibility as assessed quantitatively by LDH assay and morphologically by SEM. These results were further corroborated with plasma recalcification studies. Thus elastin-based tissue engineered scaffolds would be an attractive biomaterial for vascular graft applications.

One of the interesting observations made from the present study was that plasma proteins drastically affect platelet response to a biomaterial. In order to further elucidate these effects, fibrinogen adsorption on elastin and collagen-coated substrates was studied. Though our results did not show any observable differences, possible explanations for this phenomenon could be provided by investigating the differences in plasma protein adsorption onto different materials. Future studies could be performed with other critical proteins such as vWF, globulins and also at lower concentrations of plasma proteins to prevent substrate saturation.

Maintaining a patent endothelial lining is known to improve vascular graft performance. However in the current study it was seen that retention of seeded

endothelial cells on these elastin scaffolds was poor under shear when endothelial cells were not allowed to attach prior to applying shear stress. Thus, static endothelial seeding should be pursued *in vitro* so that endothelial cells will have time to attach and proliferate prior to putting them in fluid flow. We also investigated integrin-mediated adhesion of endothelial cells to elastin and collagen scaffolds. Among the subunits studied, both αv and β1 contributed significantly to endothelial adhesion on purified elastin substrates. Further studies on encouraging receptor-specific adhesion through pre-stimulation could be carried out for successful endothelialization of elastin scaffolds *in vitro* prior to implantation. Apart from enabling adhesion, ability of these scaffolds in supporting endothelial cell proliferation has to be evaluated, since cellular proliferation is essential to maintain a confluent monolayer at the luminal surface.

Finally, in order to successfully translate the use of purified elastin scaffolds into clinical applications, *in vivo* hemocompatibility and functional efficacy has to be assessed with appropriate animal models. Also, long-term evaluation of the tissue engineered scaffold in facilitating host tissue remodeling and regeneration has to be performed, which is the ultimate goal of a living vascular substitute.

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